Cells with mutated adenomatous polyposis coli (APC) and their sensitivity towards alkylating and processing contaminants – apoptosis and DNA damage

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MASTER THESIS IN TOXICOLOGY

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Foreword

The work that is presented in this master thesis is accomplished at the Norwegian Institute of Public Health, Division of Food, Water and Cosmetics (MIVM), in the time period from 2011- 2013. My supervisors have been Trine Husøy PhD. Steinar Øverebø PhD from the University of Oslo has served as my inside supervisor, and later he was replaced with Ketil Hylland PhD, also from the University of Oslo.

First, I would like to thank everyone at MIVM for always being helpful, cheerful and pleasant, especially Tone Rasmussen, Hege Hjertholm, Anja Hjertemo Sæther and Camilla Svendsen. The lab people at MILS also deserve a thank you for helping with Western blotting and with flow cytometry when needed.

A special thanks to Hildegunn Dahl, for much encouraging and motivational words, and for always taking time to answer my questions. This was much appreciated! Also, a special thank Camilla Svendsen for helping me with statistics. I also have to thank Trine Husøy for always being positive and giving med good advice! Last, but not least, thank you to all my friends and family!

Taken together I am grateful for the two years at the Norwegian Institute of Public Health, where I feel like I have learn a lot from nice people with a lot of knowledge.

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Abstract

Cancer is a leading cause of death worldwide, and a mutation in the APC gene is an early event in the cancer progression leading to familiar adenomatous polyposis (FAP). Mutations in both alleles of APC will eventually lead to cancer, but the time of tumor development is dependent were on APC gene the mutation is located.

Adenomatous polyposis coli (APC) is a tumour suppressor gene, with many critically functions in cellular mechanisms. In the Wnt pathway it has been shown, that a mutations in APC leads to accumulation of B-catenin. During cell migration, during mitosis, APC coordinate microtubule and organize actin. The APC gene also modulates the long-patch base excision repair (BER) pathway, possibly changing the level of DNA damage after exposure to alkylating agents. Alternatively, the cells will enter apoptosis as a consequence of extensive DNA damage. There are many alkylating agents as well as food processed contaminants, that can either induce apoptosis or affect genotoxicity.

In this thesis human embryonic cell were used. The cell line was heterozygous for the wild-type APC gene (HEK-293K), and was used as a control cell line. The other cell line has a truncated APC gene with a mutation leading to a protein expressing only the first 750 amino acids of the APC gene (HEK-293 N750).

In this study the effect of methyl methanesulphonate (MMS) on apoptosis in HEK-293K cells and HEK-293 N750 cells was investigated using flow cytometric analyses, as well investigating the effects of the mutagenic food processing contaminants furfuryl alcohol (FA) and glycidamide (GA) on genotoxicity, using the comet assay.

HEK-293K cells and HEK-293 N750 cells exposed to MMS did not increase the level of apoptosis in any of the cell lines. Western blotting was also performed on both cell lines, exposing with the alkylating agent MMS and see if cleaved- caspase-3 was detected, which is an indicator of apoptosis. No cleaved-caspase-3 was detected.

For investigation on the genotoxic effects of FA and GA, the comet assay was used. FA did not give any increase in DNA damage at any of the doses ($p= 0.992$) or exposure times tested ($p= 0.473$). There were no differences between the cell lines. However, GA gave a dose-

dependent increased in DNA damage ($p < 0.001$). When including formamido pyrimidine N-glycosylase (FPF) enzyme treatment, even more DNA damage could be detected. This showed that FPG affects the treatment of glycideamide ($p < 0.001$).

In conclusion, there were no indications that HEK-293K N750 cells were more sensitive towards MMS, and no apoptosis was observed. FA did not increase DNA-damage, but GA gave a dose-dependent increase in DNA-damage. The HEK-293 N750 cell line was not more sensitive towards DNA damage, induced by GA, than HEK-293K cells.

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Abbreviations

AA: Acrylamide

Ab-1: Anti-APC

APC: Adenomatous polyposis coli

BER: Base excision repair

BSA: Bovine serum albumin

CPT: Camptothecin

CIN: Chromosomal instability

DSB: Double strand breaks

DMEM: Dulbecco's Modified Eagle Medium

EDTA: Ethylenediaminetetraacetic acid

FA: Furfuryl alcohol

FAP: Familial adenomatous polyposis

FCS: Fetal bovine serum

FPG: Formamido pyrimidine N-glycosylase

GA: Glycidamide

HEK-293K: cell line heterozygous for wild- type APC (control cells)

HEK-293 N750: Cell line with the N750 mutant APC

HRP: Horse raddish peroxidase

MMS: Methyl methanesulfonate

PBS: Phosphate-Buffered Saline

P+S: Penicillin + streptomycin

RO: Ethyl 7-okso-7h-tieno[2,3-A]-quinolisin-8-carboksylate

TBS: Washing buffer solution

TOP1: Topoisomerase 1

1. General introduction

People with FAP have a mutation in the APC gene, and are therefore prone to develop colon cancer (Tominaga, Nita et al. 1998). The APC gene product has been found to play essential roles in many important cellular functions, such as the Wnt signaling pathway, microtubule and BER (Senda, Iizuka-Kogo et al. 2007). Impair or damage in these functions will lead to cancer, unless cellular defense mechanisms, like apoptosis, occur. Therefore it is interesting to investigate the association between HEK-293K cells and HEK-293 N750 cells. This can be done by using the alkylating agent model compound MMS.

Food processing contaminants have been widely studied, like for example furan, 5-hydroxymethylfurfural, and heterocyclic amines. Such compounds are of interest to study since many of them are possible carcinogens. Many of the food processed contaminants are themselves reactive, or form reactive metabolites, which gives genotoxic effects in the cells (Preussmann 1986). Since APC is essential for many cellular processes it is interesting to see if HEK-293 N750 cells are more prone to genotoxic effects. But so far only a limited number of dietary carcinogens have been identified and studied. This study investigates the genotoxic effects of FA and (GA).

1.1 Food processing contaminants: acrylamide and FA

Most of the food we eat is processed, prepared or preserved for storage in some way, which have advantages such as preventing growth of fungi, bacteria and other microorganisms. However, during such processes new substances may be formed, which were not present in the raw material. Many of these substances have been identified, and some shown to be hazardous and even carcinogenic to humans. They are often referred to as food processing contaminants (Preussmann 1986). Acrylamide (AA) and FA are two examples of food processing contaminants.

AA is a typical compound that forms naturally in carbohydrate rich food upon heating through the Maillard reaction, at temperatures from around 120 °C and above. This reaction also contributes to colour and flavor of the food and is a complex series of non-enzymatic reactions between an amino acid, primarily asparagine, and a reducing sugar such as fructose

or glucose. Typically AA is formed during frying, baking, and roasting and is found in crackers, breads and breakfast cereal (Mucci and Wilson 2008).

AA is neurotoxic in humans and a carcinogen in animals. Therefore it is classified as a probable human carcinogen by IARC (group 2A) (IARC 2000). Animal studies show that AA may be biotransformed to the epoxide GA by the CYP450 2E1 (CYP2E1) enzyme (Figure 1). This compound is thought to be more reactive than acrylamide itself (Kraus, Rokitta et al. 2013).

Animal studies show that AA, or its metabolite GA, cause genotoxic effects in animals and in cells *in vitro* (Knaap, Kramers et al. 1988, Ghanayem, McDaniel et al. 2005, Mei, Hu et al. 2008).

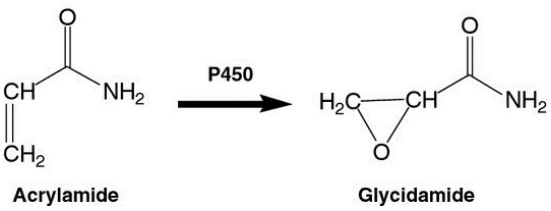


Figure 1. Biotransformation of acrylamide to GA by P450 (Gamboa da Costa, Churchwell et al. 2003)

Both AA and GA are electrophilically reactive substances, and are able to bind covalently to nucleophilic sites of proteins and DNA, where adducts are formed inducing DNA strand breaks (Doroshyenko, Fuhr et al. 2009). Detoxification happens during phase 2 metabolism by conjugating with glutathione (GSH) for urinary excretion (Fennell, Sumner et al. 2005)

The food processing compound FA belongs to the substance group substituted furans which are furan derivatives consisting of the furan ring with different side-chain attachments. FA consists of a hydroxymethyl group and a furan ring.

Furan is a compound consisting of a five-membered ring with four carbon atoms and one oxygen atom. Furan itself is genotoxic and carcinogenic in rodents, and is considered by IARC (1995) to be possibly carcinogenic to humans (group 2B).

FA is much less studied than furan, and most experiments performed with FA are inhalation experiments. Little is known about the potential hazard from oral exposure and concentrations in foods. However, FA is negative or only weakly positive in several conventional genotoxicy tests (Shinohara, Kim et al. 1986, Aeschbacher, Wolleb et al. 1989).

FA is mainly oxidized to furfural. A 2-year gavage study on the carcinogenicity of furfural was published by the National Toxicology in 1990 (National toxicology program 1990). In rats, there was some evidence of carcinogenic activity, but only in males. There was clear evidence of carcinogenicity for male mice, based on increased incidences of hepatocellular adenomas and hepatocellular carcinomas. For female B6C3F1 mice there was some evidence of carcinogenic activity, based on increased incidences of hepatocellular adenomas (National toxicology program 1990). FA is then further oxidized to furoic acid and then conjugated with glycine to form furoylglyocine (Nomeir, Silveira et al. 1992) A recent study showed that small amounts of FA are bioactivated to a mutagenic compound, 2-sulfooxymethylfuran in *Salmonella typhimurium* strains expressing human sulfotransferase 1A1 and in FVB/N mice (Monien, Herrmann et al. 2011). This indicates that FA might need the presence of sulfotransferases to be activated. Standard genotoxicity tests have low levels of or completely lack sulfotransferases.

1.2 Colon cancer

1.2.1 Cancer incidence worldwide and in Norway

Cancer ranks as one of the leading causes of death in the world. Colon cancer is the third most commonly diagnosed cancer, and the third leading cause of cancer death (Siegel, Naishadham et al. 2013)(Figure 2). This is the case for both men and women, with about 1.2 million new cases recorded annually (Ferlay, Shin et al. 2010). In Norway, there has been a stable increase of colon cancer during the last 50 years (Cancer Registry 2010 [website]).

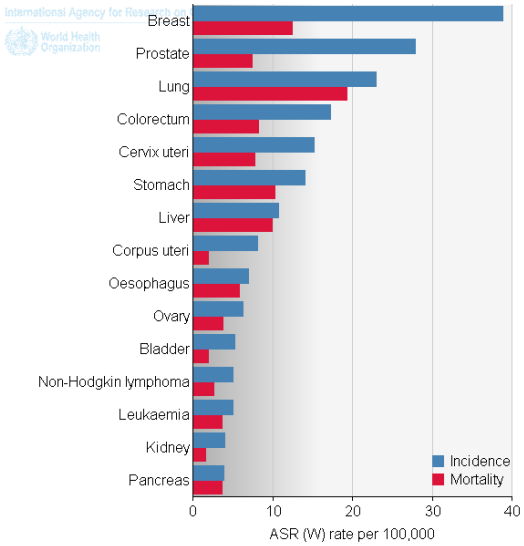


Figure 2. Estimated age-standarised insidence and mortality rates. Colourectum cancer has the fourth highest incidence rate and mortality rate (Ferlay, Shin et al. 2010).

1.2.2 Colon cancer and diet

New data indicate that the risk of developing colon cancer is associated with environmental factors, such as diets and especially the Western diet (Cummings and Bingham 1998). Studies have shown that meat, in particular meat that has been well cooked, is a risk factor for colon cancer (Xu, Yu et al. 2013). There have also been studies that indicate that smoking, reduced activity, sedentary behavior and high consumption of fatty food, fatty sugar and alcohol is associated with increased risk of health disease (Gingras and Beliveau 2011). Populations moving from low to high risk areas adopt the higher risk level of the new area (Boyle and Levin 2008).

1.2.3 Development of colon cancer

Colon cancer develops in the colon or the rectum, and usually over a period of 10 to 15 years (Siegel, Naishadham et al. 2013). Tumour progression is driven by a sequence of randomly occurring mutations and epigenetic alterations of DNA that affect the genes controlling cell proliferation, survival and other traits associated with the malignant cell phenotype, and progress through a series of histological distinct stages (from early adenoma to metastasis) (Lao and Grady 2011). Oncogenes and tumour suppressor genes are found to be the type of genes that are frequently mutated, like for example the APC gene. When oncogenes become

mutated a certain function is turned on, whereas a mutation in a tumour suppressor gene leads to loss of function (Hunter 1997, Yeo 1999).

The tumour normally begins as a noncancerous polyp (a growth of tissue that starts in the lining and grows into the center of the colon or rectum), and can develop to become cancerous. Adenomas are the kind of polyps that most likely become cancers (Siegel, Naishadham et al. 2013). The prognosis is highly dependent on the tumour stage (0 to IV) (Figure 3) at the time of diagnosis (Agesen, Sveen et al. 2012).

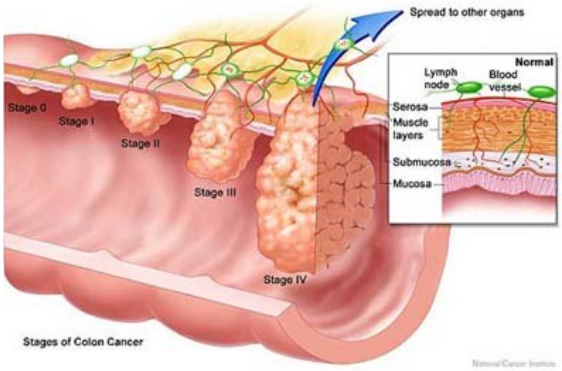


Figure 3. The overlaid stages (0-IV) of colon cancer, beginning with formation of an early adenoma (0), and finally ending in metastasis (IV) (The Daniel L.Edelstein Fund for the John Hopkins Colon Cancer Center [website]).

1.2.4 Hereditary and spontaneous colon cancer

The majority of colon cancers appear to be sporadic, but 3-5 % of the colon cancer cases in humans are caused by inherited syndromes (Narayan and Roy 2003). Examples of inherited colon cancer syndromes are the familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), as well as defects in the mismatch repair system (Narayan and Roy 2003).

The best understood heritable colon cancer syndrome is FAP, where the patients typically develop colon cancer at an average age of 42 years. Development of other tumours, including intra-abdominal desmoid tumours, osteomas, duodental adenomas and gastric adenomas may occur (Arends 2013).

FAP is caused by germ- line mutations in tumour suppressor APC (Tominaga, Nita et al. 1998). Since one allele already has damage due to mutation, the remaining allele will be vulnerable towards damages such as environmental impacts. Both APC alleles must be mutated for disease to develop.(Kinzler and Vogelstein 1996).

This gives rise to the susceptibility to develop hundreds to thousands of adenomatous polyps in the colon (Figure 4). Such polyps themselves non-malignant, but are prone to develop into carcinomas. This is due to the large number of adenomas that developed, and together with their early onset, there is a 100% risk of colon cancer formation from one of the adenomas. There has been shown, by use of genetic analysis, that the position of the inherited mutation within the APC gene affect the number of adenomatous polyps and average age of cancer onset (Arends 2013).

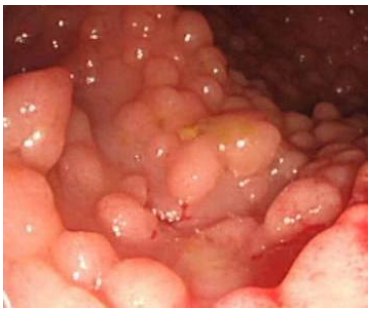


Figure 4. The overlaid stages (0-IV) of colon cancer, beginning with formation of an early adenoma (0), and finally ending in metastasis (IV) (The Family History of Bowel Cancer Clinic [website]).

Colon cancer is most frequently a result of mutations in the tumour suppressor APC gene, where CIN is associated with this mutation (Arends 2013). Mutations in the APC gene are inactivating mutations found in about 80% of all colon cancers, both sporadic and inherited, and in both adenomas and carcinomas. This occurs early in the sequence of colon cancer development (Neufeld 2009).

1.3 APC

1.3.1 APC gene and its product

The APC gene is a classical tumour suppressor gene located on chromosome 5q21 locus that codes for the 310 kDa- homodimeric multifunctional APC protein. The protein comprises an 8538-bp open reading frame and encodes the 2843 amino acid (Brocardo, Lei et al. 2008, Qian, Sarnaik et al. 2008). The structure of the protein can be seen in figure 5.

APC is normally expressed in nonproliferating colorectal epithelium, and is localized both in the cytoplasm and nucleus (Narayan and Roy 2003). The protein comprises a number of functional domains, motifs and other signatures, and can therefore perform many cellular functions through its different binding partners, besides tumour suppression. Some of these functions are control cell proliferation in the epithelium of the colon, cell differentiation, cell adhesion, chromosomal segregation, migration, apoptosis and neuronal functions (Senda, Iizuka-Kogo et al. 2007, Polakis 2012)

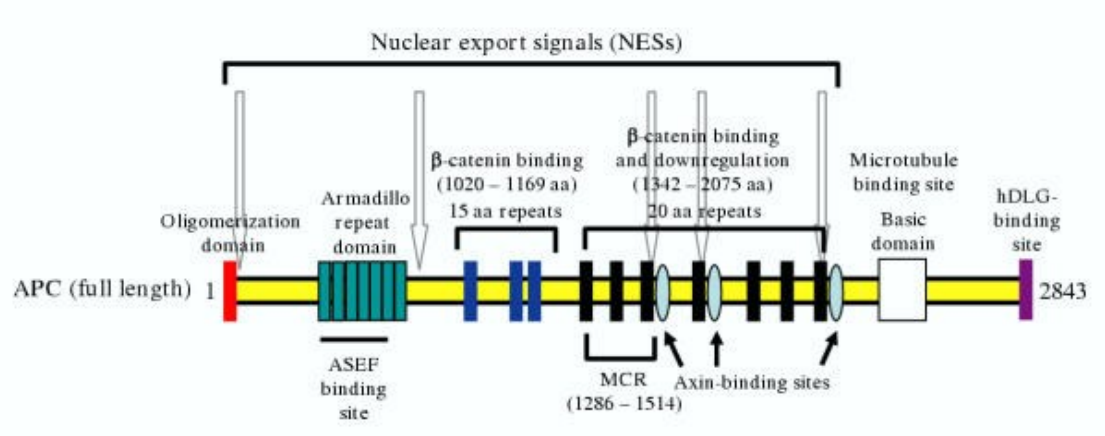


Figure 5. Structure of the human APC protein. The protein contains multiple domains (Narayan and Roy 2003).

1.3.2 APC and the Wnt pathway

One of the most studied functions of APC is its role in the Wnt signaling pathway. Loss of both alleles will lead to cancer, since the Wnt pathway is always active. This signaling pathway directs cellular proliferation and differentiation, as well as cellular morphology, motility and the fate of embryonic cells (Narayan and Roy 2003, Senda, Iizuka-Kogo et al. 2007, Polakis 2012).

In the bottom of the colon crypts APC is hardly expressed, but there are high levels of B-catenin. Upwards in the crypt the expression of APC increases, and here APC act as a regulator of the Wnt signaling pathway by negatively regulating the levels of B-catenin in the cytosol (Senda, Iizuka-Kogo et al. 2007).

APC opposes Wnt signaling by acting in a complex with axin and glycogen synthase kinase (GSK3β) to target the transcription factor β-catenin (McCartney and Nathke 2008). The binding of β-catenin is done through APC's 15 aa repeats, whereas the seven motifs of 20 aa also bind β-catenin and facilitates down regulation of β-catenin (Luchtenborg, Weijenberg et al. 2004). Thereby β-catenin becomes phosphorylated, targeted for ubiquitination and degraded by the proteosome, and controlled cell growth is maintained (Neufeld 2009).

Mutations in the APC gene can cause premature termination of translation of the APC protein, and generate a short APC protein, which lack domains that are important for its ability to associate with β-catenin and axin. This reduces the degradation of B- catenin, and B-catenin escapes its degradation through the Wnt signaling pathway and becomes stabilized in the cytoplasm. This has a profound effect on the cell, as it allows accumulation of B-catenin, followed by migration to the nucleus where it actively transcribes cell cycle related genes causing cellular proliferation (Giles, van Es et al. 2003, Narayan and Roy 2003). Cascades of events are thereby activated, leading to tumour genesis. The mechanisms described in this and the previous section can be seen in figure 6.

Alternations in B-catenin regulation are very common in human tumours. The accumulation of β-catenin is probably the most and important consequence of APC inactivation, which can be observed in about 90% of sporadic colon carcinomas (Giles, van Es et al. 2003).

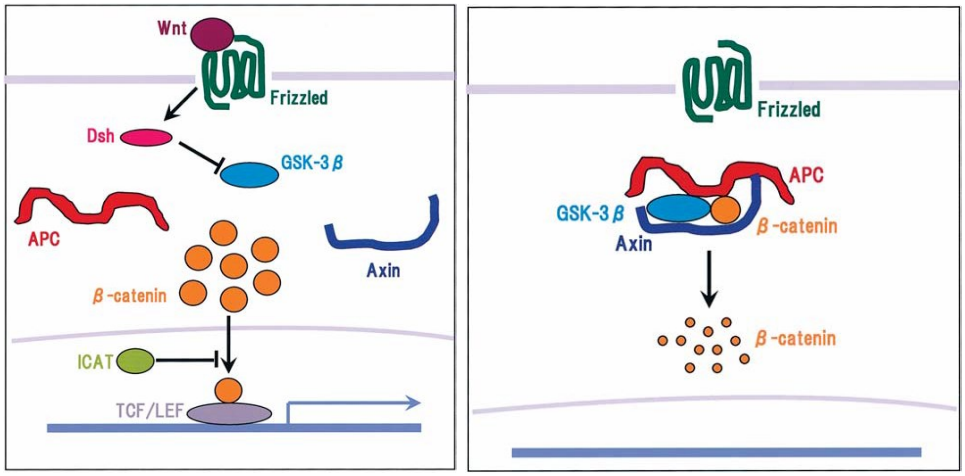


Figure 6. A simplified illustration of the Wnt signaling pathway. To the left: When Wnt is bound to the receptor on the cell surface, GSK- 3β will be inactivated. As a consequence, the complex with APC will not form and B-catenin will not be degraded. B-catenin will instead accumulate in the cytoplasm, migrate to the nucleus and bind to a transcription factor where genes that are important for cell growth are turned on. To the right: B-catenin is degraded when bound to GSK-3β/Axin/APC complex (Senda, Iizuka-Kogo et al. 2007).

1.3.3 APC and microtubuli

Mitosis is the part of the cell cycle where the nucleus is divided and is immediately followed by cytokinesis. Mitosis can be broken down to five stages: prophase, promethaphase, metaphase, anaphase and telophase. Many events of mitosis depend on the mitotic spindle, which plays an essential part in the first four stages. This structure consists of fibers made of microtubules and associated proteins. The elongation of the microtubule happens by incorporating more subunits to the structure.

The assembly of spindle microtubule starts at the centrosome, which replicate and move apart. During this process the spindle microtubule begins to form. In the promethaphase the microtubule and its plus ends are attached to the kinetochores (Green and Kaplan 2003).

During metaphase the spindle structure becomes completed and moving toward opposite ends of the cell, during anaphase, can separate the chromatids. Motor proteins associated with the kinetochore microtubules perform movement of chromosomes.

There are many proteins associated with the correct segregation of chromosomes during mitosis, but this is still under study (Cheeseman, Drubin et al. 2002). The APC protein can bind to microtubule and also interact with a number of microtubule associates proteins. It has been shown that APC is localized near the distal ends of microtubule (Mimori-Kiyosue, Shiina et al. 2000, McCartney and Nathke 2008). The multifunctional APC protein thereby plays an important role by coordinating microtubule and organizing actin during cell migration (Buda and Pignatelli 2011).

APC contains multiple domains that facilitate binding with other proteins, for example tip-binding proteins (for example KAP 3, EB1, mDIA or Asef) at the end of microtubule (Watanabe, Wang et al. 2004, Wen, Eng et al. 2004). Binding of APC to those proteins assists the movement of APC along microtubule, where APC accumulate at the plus ends of microtubule. This drives the formation of membrane protrusions, which direct cell movements (Buda and Pignatelli 2011). Binding to some of the plus-end binding proteins are needed for accurate chromosome segregation, and contribute to the movement of APC along the microtubule (Senda, Iizuka-Kogo et al. 2007). The ability APC has to localize the plus ends of microtubules contributes to stabilization of microtubule (Tighe, Johnson et al. 2004). During interface APC is also localized to centrosomes and to kinetochores in a microtubule dependent manner during mitosis (Tighe, Johnson et al. 2004).

When thinking of the many roles APC plays during mitosis it is not surprising that loss of APC leads to changes in cell migration, cell orientation, cell polarity and cell division. Mutant APC has been shown to predispose cells to increased mitotic abnormalities (Arends 2013). High rates of chromosomal instability (CIN) have been reported as an underlying cause of colon cancer (Green and Kaplan 2003). There has been shown that in Xenopus egg extracts depleted of APC protein changes in microtubule density occured, resulting in the formation of weaker spindle. Whereas in human cells with APC mutations there has been reported aberrant spindle structures and weakened kinetochore (Green and Kaplan 2003, Dikovskaya, Newton et al. 2004). There has been shown that truncated APC proteins can result in chromosome instability, which can drive the accumulation of mutations, including loss of the second APC allele in FAP patients (Tighe, Johnson et al. 2004). In cells expressing N-terminal fragments

of APC similar effects have been observed (McCartney and Nathke 2008). In cells heterozygous for Apc ^{min} mutation in Apc show aberrant chromosome segregation, which leads to CIN (Husoy, Cruciani et al. 2003).

1.3.4 APC and DNA repair

The structural integrity of DNA is continuously subjected to a number endogenous processes and exogenous substances, as well as to bi- products from the cell´s own metabolism (reactive oxygen species), which lead to DNA damage. Such influences can affect the maintenance of the genomic integrity, and potentially lead to mutations and be a reason for carcinogenesis if not repaired. Cells possess several defense mechanisms to repair different kind of DNA damage and to eliminate damaged cells. Several different repair pathways exist, which are activated upon DNA damage. These pathways stall the cell cycle progression and thereby provide more time for repair of the damage (Bartek, Bartkova et al. 2007).

The major repair pathways in mammals are excision base repair (nucleotide excision repair and base excision repair) and recombination repair (homolog recombination and non-homolog recombination).

The APC gene can modulate the long-patch base excision repair (BER) pathway when there is too much damage. APC levels increase, following blocking of DNA- polymerase β (Pol- β) and flap endonuclease 1 (Fen-1), which thereby prevent the pathway (Balusu, Jaiswal et al. 2007, Jaiswal and Narayan 2008). However, these effects on the cells ability to repair are not fully understood.

If the damage is too significant for the cell, DNA repair will not be possible. Defects in these mechanisms make the cells more sensitive toward DNA damaging agents, which may have serious consequences, leading to disease and cancer predisposition (Jackson and Bartek 2009).

1.5 Apoptosis and necrosis

Based on the cell´s proliferative status, capacity of repair enzymes and ability to induce proteins that promote cell death, a cell can undergo different types of cell death if there is too much DNA damage (Figure 7)(Okada and Mak 2004). Only apoptosis and necrosis will be mentioned.

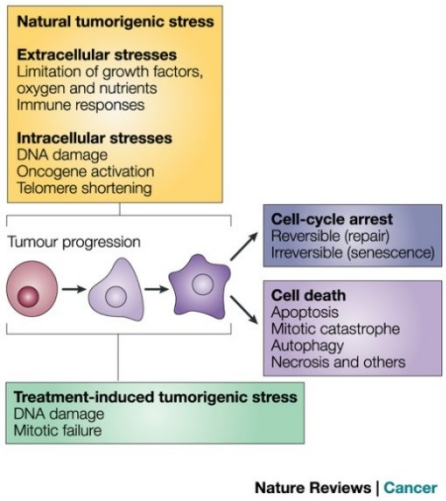


Figure 7. Different responses a cell has towards stress (Okada and Mak 2004).

Apoptosis is programmed cell death that requires adenosine triphosphate (ATP), and is a well-defined process with characteristic morphology (Kerr, Wyllie et al. 1972).

In multicellular organism, apoptosis is essential for normal cell development, differentiation, homeostasis and embryogenesis. Apoptosis is especially important when it comes to regenerating of tissues, for example in the crypt-villus intestinal structure where there is apoptotic elimination of cells at the tip of the villus (de Jesus Perez, Yuan et al. 2012).

Apoptosis serves to eliminate old cells, cells having undergone DNA damage, cells being infected and inappropriately proliferating cells (Leist and Jaattela 2001, de Jesus Perez, Yuan et al. 2012).

The mechanisms behind apoptosis are mediated by caspases, a family with cysteine proteases (Kouzmenko, Takeyama et al. 2008). Two major pathways trigger caspase activation and

apoptosis; the death receptor pathway and the mitochondrial pathway, where initiating events and downstream events distinguish them (Tang, Mura et al. 2008).

Casapses are expressed as inactive pro-caspases precursors, which undergoes proteolytic activation. Caspases are grouped into initiators (caspase-2, -8, -9 and -10) and effectors (caspase-3, -6 and -7). The first group is activated in response to particular cell death and developmental signals, and cleaves the precursors forms of the effector caspases. The effector caspases become activated and execute apoptosis by cleaving other proteins necessary for cell survival (Tang, Mura et al. 2008). Active caspase- 3 is a protein that gets activated during the early phase of apoptosis, and starts when caspase- 3 gets cleaved into a shorter protein (Shrivastav, De Haro et al. 2008).

Caspases digest designated cellular proteins, which leads to breakdown of the cell down to apoptotic bodies, without disrupting the cellular membrane (Figure 8) (Jaattela 2004). The apoptotic cells are recognized by changes in the plasma membrane and phagocytized by cells of the immune system. This prevents leakage of cellular content of the surroundings and is considered less inflammatory (Fadeel and Orrenius 2005).

If apoptosis fails accumulation of cells will occur, resulting in abnormal cell proliferation, where cancer is the worst outcome (Bratton and Cohen 2001).

Necrotic cell death is often a passive process, and is frequently a result of ATP depletion. It is characterized by irreversible increased cell volume, organelle swelling, loss of plasma membrane integrity and intracellular content (Figure 8) (Asare, Landvik et al. 2008, Tang, Mura et al. 2008). This releases cellular components, affecting the surrounding cells causing inflammation (Fadeel and Orrenius 2005).

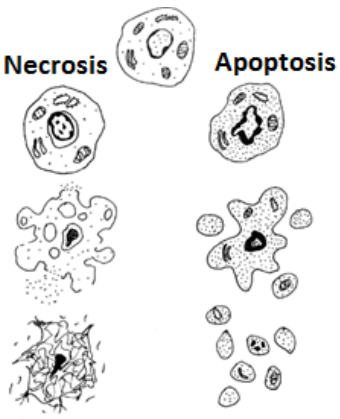


Figure 8. An overview of apoptosis and necrosis. Necrosis is characterized by swelling and rupture of the cell membrane, and apoptosis is characterized by several apoptotic bodies (Robertson and Orrenius 2000).

1.6 Alkylating agents

Alkylating agents are a class of anticancer drugs that bind to DNA and prevent proper DNA replication. These compounds also occur as mutagens and carcinogens in the environment (Peto, Gray et al. 1984).

The simplest alkylating agents, primarily methylating and ethylating agents, are not normally considered an environmental contaminants, but are used as model compounds for reactive forms of many carcinogens (Beranek 1990). MMS is such a model compound, and has been used for a long time as an alkylating agent that causes DNA damage, and gives double strand breaks (DSB) (Lundin, North et al. 2005, Benchabane and Ahmed 2009). MMS modifies guanine (to 7-methylguanine) and adenine (to 3-methyladenine), causing mispairing and replication blocks, respectively, as well as it can give rise to DSB. When there are DNA repair pathways that are compromised, cells exposed to MMS show an increased sensitivity (Lundin, North et al. 2005).

Another anticancer drug is the alkaloid camptothecin (CTP), which intercalates between DNA bases and amino acid residue. It is a highly selective topoisomerase I (TOP1) inhibitor, where inhibition of this enzyme impairs transcription and DNA replication, resulting in DSB in the DNA, and thereby hindering cellular processes (Tomicic and Kaina 2013).

1.7 Flow cytometry

Flow cytometric measurements are widely used for analyzing different cell types in a solution of heterozygous population, where multiple parameter analysis of a single cell can be determined.

Flow cytometric measurements can also discriminate cells in the different parts of the cell cycle: the G1-, S-, G2- and M-phase. Distribution of cells in the major phases of the cell cycle is based on differences in DNA content. Since G2- and M-phase have identical DNA content flow cytometric measurements cannot discriminate between the two. Discrimination is based on DNA content (for example by Hoechst 33258 fluorescent), cell size (forward scatter) and cell granularity or internal complexity (side scatter). When using flow cytometry to analyze apoptosis, apoptotic cell bodies will emit fluorescent signals that are lower than those of G1 because of chromosome condensation and nuclear fragmentation, and are therefore called sub-G1 (Wiger, Finstad et al. 1997).

Cells are prepared from a sample, stained with different fluorochromes (that attach to proteins or DNA), and passed through a laser and optic detection system. A sheat fluid ensures that only one cell at the time passes the detection system, with a great capacity (>300 cells/second) to acquire and analyze. However, samples should be analyzed at rates below 1000 cells per second in order to yield a good signal of discrimination between singles or doublets. Cells of interest in a population are gated and doublets are discriminated. Diverse software can be used to fit a DNA histogram where percentages of cells occupying different phases of the cell cycle are calculated(Otsuki, Li et al. 2003).

1.8 Western blotting

Western blotting is a widely applied analytical technique used for separation and identification of expression levels of proteins, and was first described by Towbin and colleagues (Towbin, Staehelin et al. 1992). In order to separate proteins in a cell lysate, samples prepared from biological material are diluted and cooked in a buffer containing sodiumdodecylsulfat (SDS), mercaptoetanol, glycerol and bromphenol blue. Cooking of the sample at 95°C breaks the secondary and tertiary structure of the proteins, and

mercaptoethanol ensures that the proteins do not form these structures again thereafter. SDS gives the proteins a negative charge, bromphenol blue gives colour to the protein lysate and last but not least, but not least, glycerol ensures the right viscosity. Equal amounts of proteins are separated by electrophoresis, using polyacrylamide gel. Electrophoresis is performed in an electrophoresis tank filled with buffer that can carry current. Proteins with negative charge will migrate away from the anode. With electrophoretically transfer the proteins are moved from the gel and onto a nitrocellulose support membrane, where they become immobilized. In order to immobilize the proteins on the membrane, the membrane is placed between filter paper, sponge pads, and on top of a nitrocellulose membrane as well. This sandwich-like packing is placed in a tank with buffer solution, where the transfer occurs. In order to prevent nonspecific binding the membrane is blocked (Gershoni and Palade 1983, Towbin, Staehelin et al. 1992)

The specificity of Western blotting is achieved by using antibodies (monoclonal or polyclonal) that recognizes and binds to an epitope unique to the protein of interest. Furthermore, a secondary antibody, conjugated with horse raddish peroxidase (HRP), is added and binds to the primary antibody- antigen complex. To detect the antigen-antibody complex the membrane gets incubated with chemiluminnescent. The HRP on the secondary antibody leads to an enzyme reaction with light as product, which can be detected using a film or digital imaging. To identify proteins a standard protein ladder is used. The principle behind Western analysis can be seen in the figure below (Figure 9) (Kurien and Scofield 2006).

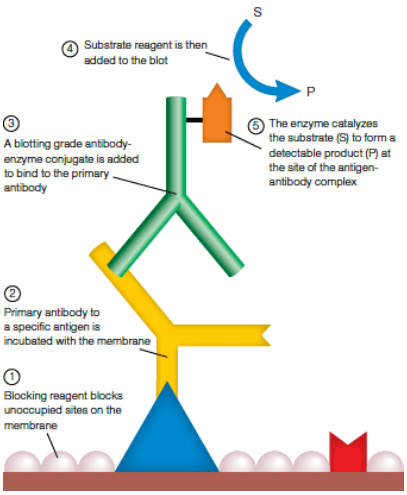


Figure 9. Principle for detection of proteins by Western analysis (BioRad [website]).

1.9 Comet assay

The single cell gel electrophoresis assay (SCGA), also called comet assay, due to the comet-like shape of nuclei formed after electrophoresis of damaged DNA (Figure 10), is a simple, effective and inexpensive method for measuring DNA damage. The comet assay can be performed under neutral or alkaline conditions, where the first one in generally more sensitive (Collins, Dobson et al. 1997). Basically, when using the alkali comet method, DSB and alkali-labile sites (ALS) can be detected. There are various specific enzymes (enzymes that cut DNA at specific locations) that can be used before the electrophoresis step to detect multiple DNA damage. The use of formamido pyrimidine N-glycosylase (FPG) is wieldy used, and is recommended for the detection of oxidative DNA basa damage. It removes oxidized purines and formamidopyrimidines, and also attacks ring-opened N7 guanine adducts produced by alkylating agents (Li, Laval et al. 1997) .

Single cell suspension is prepared from cell culture (in vitro) or from the tissue (in vivo). The cells are embedded in agarose-gel, on a film, and lysed. After lysis of the cells overnight, the electrophoresis step is performed. The double strand break DNA will migrate (towards the anode) to form a comet-like tail. The size of the comet tail will be proportional to the amount of DNA damage (Gutzkow, Langleite et al. 2013) Azqueta and Collins 2013).

This method is very sensitive, allowing a few thousand of lesions in each cell to be detected; it is relatively simple and cost-efficient, and a very low number of cells may be used.

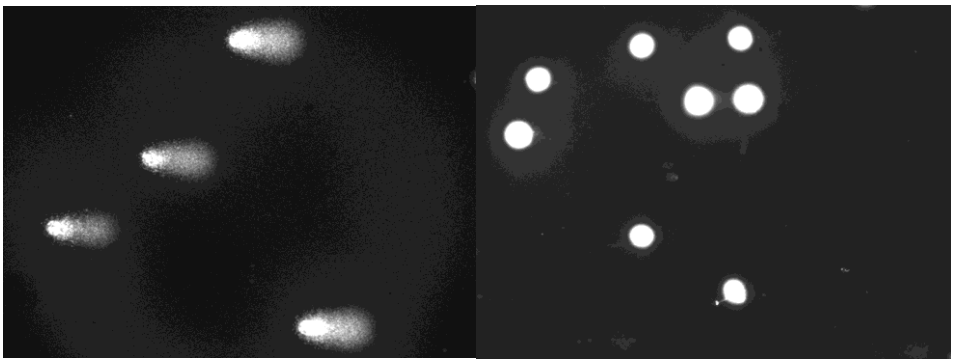


Figure 10. Damage cells (to the left) look like a "comet" under the microscope, compared to undamaged cells (to the right) (own picture).

1.10 Aims of study

By using cells with a normal functioning APC protein (HEK-293K) and a truncated form (HEK-293 N750) of the protein, one aim of this study was to explore the effects of MMS on apoptosis.

1. Is there any likelihood that cells with mutated APC are more sensitive and undergo apoptosis when using MMS?

A second aim was to clarify effects of the mutagenic food compounds FA and GA.

2. Will exposure to FA or GA cause increased DNA damage?
3. Is more DNA damage observed for GA, when using FPG?
4. Will cells with mutated APC be more sensitive towards mutagenic compounds?

2. Materials and methods

Information about products, instruments, software and producers can be found in appendix 1-3.

There are many methods available for detection of cell death. In this study apoptosis was investigated, and flow cytometry was found to be a useful method, as it can give useful information of apoptotic cells by discriminating between cell size and DNA content. Apoptotic cells have smaller size and less DNA content than the cells in the G1 phase, and can be visualized. For staining the cells, there are different types available, the Hoechst 33258 (Invitrogen) was chosen. Another reason for choosing the method was that it is efficient, allowing quantification of a high amount of cells.

Activation of different caspases by cleavage is a molecular marker for apoptosis. Caspase-3, a key player in the process of apoptosis, and cleaved-caspase-3 was investigated with Western blotting. This can give information about the activity of a protein.

The comet assay was chosen as the method for detection of DNA-damage because of its sensitivity and because only a small number of cells is needed. Another advantage is that when using FPG enzyme, more information of DNA damage can be provided.

2.1 Cell work

2.1.1 The cell line

In this study human embryonic kidney cells, HEK-293, from Great Britain was used. This is a cell line transfected with the APC gene with three different mutations, which are N750, N1309 and N1807. The mutants are expressed as Myc-proteins and they code for the first 750, 1309 and 1807 amino acid of the APC gene (Tighe, Johnson et al. 2004). These mutations cause different length of the APC protein, which gives the opportunity to study the APC protein with different lengths. This provides a good model system to study the function of APC.

In this study the cell line with the N750 mutant APC (HEK-293 N750) was used, as well as the cell line heterozygous for wild- type APC (HEK-293K), which was used as control. Both of the cell lines were used in all experiments, and they were given the same treatments.

The HEK-293 N750 cells were generated to express the Myc-tagged N-APC mutants under tetracycline control (Tighe, Johnson et al. 2004). To induce the truncated form of the APC protein, in the HEK-203 N750 cells, the medium used prior to an experiment contained tetracycline hydrochloride (0.01μg/ml). The same treatment was given to the HEK-293K cells, this was done in in order to give the two cell lines the same treatment.

2.1.2 Sterile technique

The study involved working with living cells and accuracy concerning sterile technique, which had to be performed correctly to avoid contamination and to maintain a clean and sterile working environment. The cell work took place in an approved ventilated cabinet (DanLaf) in order to maintain sterile condition as well as to prevent exposure to dangerous substances. Personal safety equipment was always used, and protective goggles if needed. The material data safety sheet was always read prior to an experiment.

2.1.3 Cell culture

The cell lines were cultivated in plastic culture flasks (75 cm^2 flask) (Starstedt) with 15 ml of the cell culture medium, Dulbecco's Modified Eagle Medium (DMEM) (VVW).

The flask was kept in a humidified incubator (Thermo Steril- Cycle CO_2 incubator) at 37°C with 5% CO_2 humidified air. The cap of the flask was always slightly opened when being in the incubator in order to ensure equilibrium of the CO_2- gas in the incubator and the cell medium.

A 500 ml flask of DMEM contained 4.5 g/l D-Glucose, L-Glutamine and pyruvate. DMEM was supplemented with 10% Fetal Bovine Serum (FCS) (Biochrom), L-Glutamine (2mM) (Sigma Aldrich) and 1% penicillin/streptomycin (P+S) (Lonza). Medium with these supplements will in the rest of this document be called medium. The HEK-293 N750 cell line was grown in medium that also contained hygromycin B (Hyg. B.)(0.2 mg/ml) (Invitrogen Life Technologies).

All medium was used at physiological temperature. Medium/Hyg.B. was made in advance because Hyg. B lowers the pH, and the pH needs to be normalized before medium/Hyg. B is being used. Since DMEM and Hyg. B. are light sensitive, these solutions were kept in the dark until being used.

The cells were passaged approximately every third day depending on when they were needed in an experiment. The process of splitting the cells involved removing the old cell culture medium with suction (Integra vacusafe) and washing the cells with Dulbecco Phosphate-Buffered Saline (PBS) (locally made). In this study PBS without Ca^{2+}/ Mg^{2+} was used.

To detach the cells from the bottom of the cell culture flask, trypsin/EDTA (170000 U/l) (trypsin) (Lonza) was added. To a big flask (75 cm^2) 1 ml trypsin (Lonza) was added, and to a small flask (25 cm^2 flask) 0.333 ml was added. The flask was put to incubation for 2 minutes, at 37°C. Detachments of the cells were controlled under the light microscope. Thereafter, medium was added to stop the trypsination, and also to obtain a cell suspension. The resulting cell suspension was placed in a 15 ml tube. After resuspending the cells, some of them were aliquoted to a new cell culture flask and put for incubation until the next split was necessary.

To obtain the right cell density for the experiments, the number of cells in the cells suspension was counted. In the beginning of our experiments a Bürkner counting chamber (Labor Optik®) was used in order to determine the concentrations of the cell suspension. The rails of the Bürkner counting chamber (Labor Optik®) were moistened with distilled water and a cover glass was placed on top of the counting chamber. Trypan blue stain (Lonza) was used to discriminate between dead and living cells. An intact cell membrane of a living cell will exclude the colour, and in cells where the cell membrane is damaged and in dead cells trypan blue to cross the membrane and stain those cells. A desired dilution of the cell suspension with medium and trypan blue stain (Lonza) was made, and 10 µl of the dilution were pipetted under the cover glass. The cells were counted under a light microscope (Nixon) using a 10X objective. The averages for both chambers were calculated, and this number was used as the number of cells per square. Thereafter the number of cells per ml was calculated.

Example:
In a 2x dilution the amount of cells per ml is calculated like this; the amount of cells per square x 1000 x 2. In a 10x dilution the amount of cells per ml is calculated like this; the amount of cells per square x 10000 x 10.

An automatic cell counter (BioRad) along with cell counting chamber slides (BioRad) was later on used to count the cells. The cells were diluted 1:1 with trypan blue stain (Lonza), and 10 µl of the dilution was added to the counting chamber slide (BioRad).

Depending on the experimental method, the proper amount of cells was taken out from the cell suspension and placed in a flask or a dish along with medium. (Table 1). The different amounts of cells used for flow cytometry depended on when they were further used. If they were used the next day a higher amount was needed, opposed to when they were used two days later.

Table 1. Different cell concentrations, containers and volumes of medium used for the different setups.

Experimental method	Amount of cells	Container	Volume of medium (ml)
Western blotting	2 x 10^6	10 cm in diameter dishes	10
Flow cytometry	15-40 x 10^6	10 cm in diameter dishes	10
Comet assay	1 x 10^6	25 cm^2 flasks	5

The dishes/flasks were placed in the CO_2 incubator until used. Before exposing the cells to a chemical, the medium was changed approximately 10 minutes before starting the treatment. In all experiments, except for the comet assay, preformed with GA, medium with supplements was used. In the experiment with GA, medium with no supplements was used in order to prevent reaction between GA and proteins in the medium.

When exposing the cells to a chemical, the chemical was added to the medium surrounding the cells. Since the amounts of the different chemicals were low (µl) no equivalent amount of dimethyl sulphoxide was added to the control cells. The exposure concentration of the different chemicals can be seen in tables 2 and 5.

2.2 Flow cytometry

2.2.1 Preparing the cells for Flow cytometry

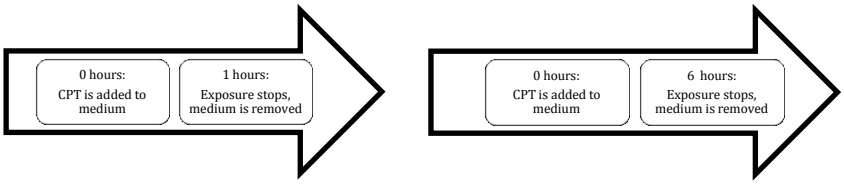
The cells were prepped for an experiment in the same way as described under the section "cell culture". The dishes were grown to near 80% confluence before treatment with a chemical (Table 2). Both cell lines got the same treatment. The experiment was carried out four independent times.

Table 2. Treatments given to the cells, along with concentrations.

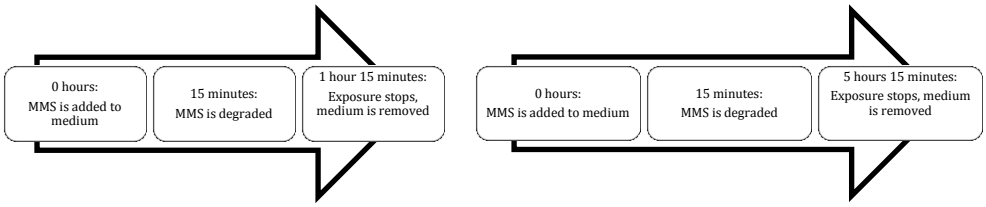
Treatment	Control	CTP and MMS	CTP	MMS
Concentration of treatment	0 mM	10 µM and 600 µM	10 µM	600 µM

The exposure duration always consists of two independent times. The duration of treatment with CPT lasted for 1 hour and 6 hours. The duration of the treatment with MMS lasted for 1hous and 15 minutes as well as 5 hours and 15 minutes. The treatment with the combination of the chemicals lasted for 1 hour and 6 hours (Figure 11). No time line for the control treatment is shown as this exposure lasts as long as the shortest exposure time.

A. CPT exposure



B. MMS exposure



C. Exposure of CPT and MMS

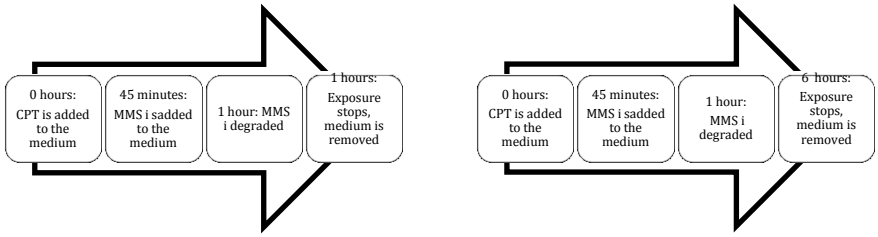


Figure 11. An overview of the treatment times and lengths. **A.** CPT time line **B.** MMS time line. **C.** Timeline for the combination of CPT and MMS

After ended exposure, old medium was removed and the cells were washed 2 times with PBS before trypsination (Lonza). Medium with 20% FCS (PAA The Cell Culture Company) was used to stop trypsination. The cell suspension was transferred to a 15 ml tube (Sarstedt) and kept on ice, until 3 minutes centrifugation at 700 rpm. The supernatant was removed, and 1 ml medium with 20 % FCS (PAA The Cell Culture Company) was used to resuspend the pellet. From the cell suspension a 500 µl sample was taken out and prepared for flow cytometry. This involved fixation of the cells, where a sample got added 2% paraformaldehyde (PFA) (Sigma Aldrich). The cell suspension was put in BD Falcon 5ml PS tubes (BD Biosiences) and stored in the refrigerator until use.

About 15 minutes before flow cytometric measurements the DNA of the cells were stained, with a 1:1 mixture of Hoechst 33258 (1 µg/ml) (Sigma Aldrich) and Trition X-100 (10%) (Thermo Scientific).

The samples were kept in the dark while not being analyzed by BD LSR II flow cytometer (BD Biosciences). Before recording data for a sample, the sample was gently vortexed.

2.2.2 Flow cytometric measurements

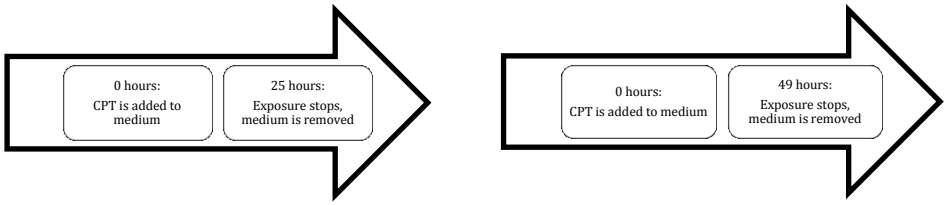
The number of events to acquire was set to 10 000. The threshold rate was regulated if needed. The blue laser (480nm) was used, and for measuring Hoechst the detector Alexa Fluor 405 was used. The G1 peak was set to 100, and the apoptotic index was determined as the percentage of signals between the G1 peak and the channel positioned at 20% of the G1 peak (sub-G1 population). FACS Diva software (BD Biosciences) was used for further analysis.

2.3 Western blotting

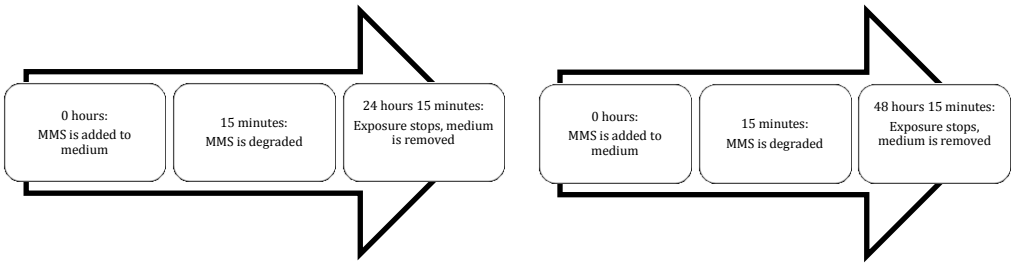
Western blotting was performed in order to check if the HEK-293 N750 cells expressed the truncated form of the APC protein, and to check for apoptosis. This was done using Anti-APC (Ab-1) monoclonal antibody (Calbiochem) for detection of the truncated APC protein in the HEK-293 N750 cells. The polyclonal antibody Caspase-3 (Cell Signaling) was used for detection of apoptosis. The cells were prepped for an experiment in the same way as described under “cell culture”, and three independent replicates of the experiments were performed.

The different treatments and treatment concentrations can be seen in table 2. The duration of the treatments are the same as in figure 11, but with an additional time scenario: 25 hours and 49 hours for CPT, 24 hours 15 minutes for MMS, and 25 hours and 40 hours for the combination of them (Figure 12). There is no time line shown for the control treatment as this exposure lasts as long as the shortest exposure time.

A. CPT exposure



A. MMS exposure



C. CPT and MMS exposure

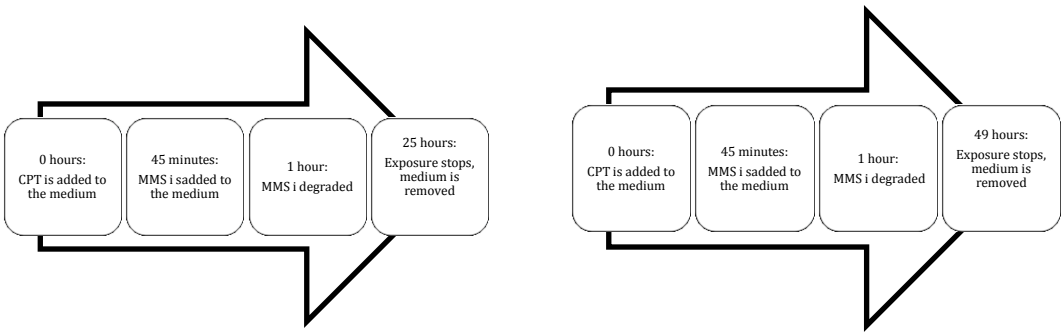


Figure 12. An overview of the treatment times and lengths. **A.** CPT time line **B.** MMS time line. **C.** Timeline for the combination of CPT and MMS

2.3.1 Harvesting cells

After ended exposure times the adherent cells were harvested on ice. Medium was removed with suction (Integra vacusafe) and PBS was gently added 2 times in the corner of the dish to rinse. PBS was removed by suction and cell paper was used to remove redundant PBS from the corners. The dishes were placed in a -20°C freezer for about 5 minutes before being stored in a -70°C freezer until further use. The dishes were placed on ice and lysates were made by adding, 200 µl of lysis buffer to each dish. A cell scraper was gently used and the lysate was transferred to an eppendorf tube and sonicated 5 pulses, each lasting one second with amplitude of 30, before the lysates boiled for 5 minutes at 95 °C. Centrifugation for 10 minutes at 12 000 rpm followed. The supernatant was moved to a new eppendorf tube, and the pellet was discarded.

2.3.2 Protein measurements

Lysat was diluted 1:10 in distilled water. The standard curve (four parametric curve) was made in distilled water and bovine serum albumin. Concentrations being used for the standard curve were: 4, 2, 1, 0.5, 0.3, 0.125 mg/ml.

BioRad DC Protein Assay (containing solution A, B and S) (BioRad) was used to measure protein amount. To a microtiterplate four replicates with 5 µl of each sample and three replicates with 5 µl of the standard curve, as well as three replicates distilled water was added. Further, 25 µl of Reagent A containing 2% of reagent S and 200 µl reagent B was added to the whole plate. The plate was placed in the dark for 15 minutes in order to obtain colour development, and absorption was measured in a plate reader at 750nm and analyzed with the software Gen 5. Lysis buffer was added to adjust the wanted protein concentration. The samples were boiled in water for about 5-10 minutes at 95°C before they were applied to the gel.

2.3.3 Electrophoresis and blotting

Proteins were separated in polyacrylamide gels (Mini-Protean TGX Precast gels) (BioRad). Depending on the protein of interest, 12.5% or Any % kDa gels were used, and each well had a total amount of protein of 12.5, 18.75 or 14.0 µg/µl (Table 3).

Table 3. The percentages of the gels being used and the total amounts of protein applied.

% of the gel	Total amount of protein	Protein of interest
Any % kDa	• 12.5 µg/µl • 18.75 µg/µl	• APC
12.5 %	• 12.5 µg/µl • 14.0 µg/µl	• Caspase- 3

Before applying the samples, the wells were rinsed with electrophoresis buffer, in order to get rid of possible rests from the buffer, from the manufacturing process. The samples were vortexed and applied to the wells. In order to allow direct visualization of the sample mobility during electrophoresis and for precise sizing of proteins, 10 µl Precision Plus Protein Prestained Standard Dual Xtra (BioRad) was added to a well. Electrophoresis was run at 100V until the samples had gathered to a straight line, and was sat up to 200V. While running electrophoresis the electrophoresis chamber was placed in a plastic box, encircled with ice.

Proteins where transferred onto a nitrocellulose membrane (Whatman®) by electro blotting. Blotting was carried out in transfer buffer for 30 minutes at 100V and then for 1 hour at 70V. The step was done with an ice block in the blotting chamber to keep the temperature low, and in order to maintain cold circulation of the transfer buffer, a magnet stirrer was used. Ponceau-staining was used to control the protein loading. Prior to probing the membrane with antibody, the membrane was blocked in washing buffer solution (TBS buffer) with 3% dry milk for at least 30 minutes, at room temperature, and with shaking. This was done in order to avoid unspecific binding. Subsequently, the membrane was washed 3 timed for 10 minutes with TBS buffer.

Different antibodies and dilutions were used in the different experiments to detect the different proteins of interest, along with a corresponding HRP-conjugated secondary antibody

(Table 4). Incubation with primary antibody, in TBS with 1% dry milk, was always preformed over night at 4 °C, and with shaking. Incubation with HRP-conjugated secondary antibody, in TBS with 1% dry milk, was always preformed at room temperature, and with shaking

Table 4. Overview of types and amounts of the primary and secondary antibodies.

Primary antibody	Dilution	Secondary antibody	Dilution
Ab-1	1:500	Anti- mouse HRP	1:5000
Caspase-3	1:1000	Anti- rabbit HRP	1:5000
B-aktin	1:200000	Anti- mouse HRP	1:5000

The next day the membrane was washed 3 times for 10 minutes with TBS, before incubation with HRP-conjugated secondary antibody (Dako). This was performed at room temperature on a shaker for 1-2 hours.

The proteins were visualized by using Super-Signal® West Dura Extended Duration substrate (BioRad), according to the manufactures protocol. Equal amounts SuperSignal®West Dura Luminol/Enhancer Solution and SuperSignal®West Dura Stable Peroxide buffer were added to the membrane and incubated in the dark for 3-5 minutes. Image Lab Analysis Software (BioRad) was used for visual quantification of the protein bands.

Finally, the membrane was re-probed with the primary antibody B-actin as a loading control. This was done in each Western experiment. This secondary antibody was also incubated in TBS 1% dry milk. Visualization was done as described above.

2.4 Comet assay

For the comet assay the cell lines were exposed with two different substances, FA and GA. For both chemicals dose-response experiments were performed with three independent replicates, and with the FA time curve two independent replicates were performed. Treatment with the FPG enzyme was included in the experiments with GA.

2.4.1 Preparing the cells for comet assay

The cells were prepped for an experiment as described under the section "cell culture". The next day the flasks were controlled under a microscope (Nixon TMS Type 104) to see if the cells had attached to the bottom of the flask. Different concentrations were used for the different chemicals, in the different experiments, and with different incubation times (Table 5).

Table 5. An overview of the concentrations of the chemicals in the different experiments. Type of experiment and incubation time is listed as well.

Chemical	Experiment	Incubation time (hours)	Exposure concentration (mM)
FA	Dose/response	3	0 | 0.5 | 3 | 10
	Time curve	0 | 1 | 5 | 7 | 24	10
GA	Dose/response (pilot)	2	0.5 | 2 | 5
	Dose/response	2	0 | 0.05 | 0.1 | 0.5

When exposing the cells with FA, the stock solution was made fresh in medium, immediately before exposure. During incubation the cap was properly closed in order to prevent the chemical to evaporate. When exposing the cells with GA, the stock solution was made fresh in PBS. The cells were incubated in the CO_2 incubator according to the times given in table 5. The further procedure was the same for both chemicals.

The exposure treatment for both compounds was terminated by removing the old medium, washing the cells 2 times with PBS, and treatment with trypsin (Lonza). Medium was added and the cell suspension was transferred to a tube and centrifuged for 3 minutes at 700 rpm. The cell pellet was gently resuspended to 10^6 cells/ml in medium, and stored on ice. The following steps were done in dim light or in the dark if nothing else is specified.

Low melting soft agarose (0.75%) (Cambrex) was dissolved, in PBS containing 10 mM EDTA (pH 7.5), heated in a microwave and stored on a 37°C heating block. Agarose was mixed with cells in a 1:10 dilution.

GelBond® film (Cambrex) were cut to size 8.5cm x 12.5cm, with a cut in the bottom right corner of the film and with holes in each of the corers (Figure 13). The film was attached to plastic frames to simplify handling between the different treatment steps following later on.
For each exposure sample 4 parallels with 4 µl agarose/cell mixture were placed onto the hydrophilic side of a GelBond® film (Cambrex) while sited on a cold plate. Immediately after embedding the gels and thereby immobilizing the cells, the films were put for lysis, using fresh lysis buffer, overnight at 4°C.

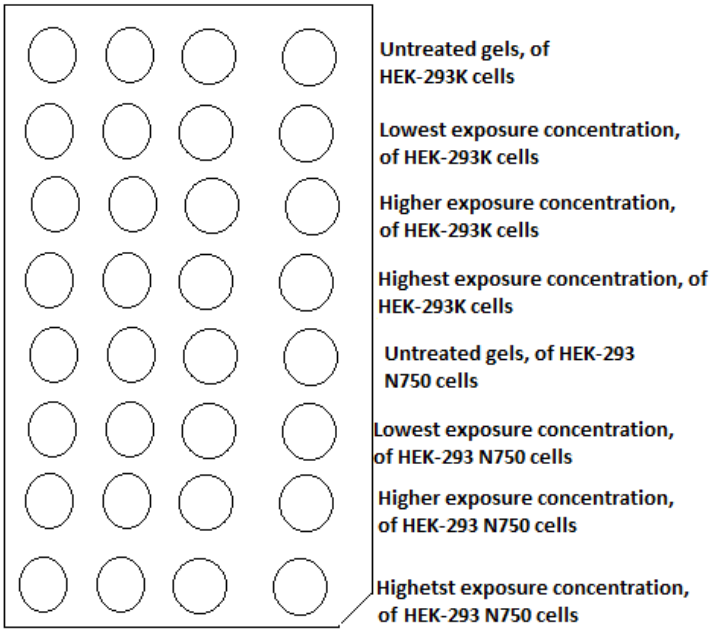


Figure 13. A typical setup on a GelBond® film (Cambrex). The picture shows the different treatments of each cell type, and where each sample is replicated as 4 parallel gels (horizontal) on the film.

The next day the films were rinsed with distilled water, and placed in electrophoresis buffer at 4°C for 5 minutes and then in new electrophoresis buffer for 35 minutes in order to unwind DNA. The film was immersed onto a platform in an electrophoresis tank, using the same solution used for DNA unwinding. Electrophoresis lasted for 20 minutes in the dose-response experiment with FA and 25 minutes in all other experiments, at 8°C. V/cm over the platform was always between 0.8-1, mA was approximately 700 and the voltage was 25. Since DNA is negatively charged it will move towards the anode, giving the appearance of a comet tale.

The next steps in the procedure took place at room temperature. The film was neutralized in neutralization buffer for 2 times for 5 minutes and then fixed in ethanol for at least 90 minutes, before it was put to dry in the dark.

For further analysis the film was re-hydrated and stained with SybrGold (1x) which was diluted in TE-buffer, for 20 minutes. Excess stain was washed off with distilled water. The film was put in a moist container and kept in the dark at 8°C for at least 10 hours for post staining.

Analysis of DNA tail migration was performed using a Leica DMLB fluorescence microscope (using 20X lens) equipped with CCD-camera, and single cells were semi-automated scored by the use of Comet IV capture system (version; Perspective Instruments, Liverpool, UK). For each gel 30 cells were randomly counted when having 4 parallels.

In the experiments done with GA, the enzyme FPG was used as well, for detection of oxidative DNA damage. Samples were both untreated and treated with FPG, which recognizes specific lesions and converts them to strand breaks. In order to determine the optimal concentration of FPG for the cells, an enzyme titration was done in the HEK-293K cell line.

2.4.2 Enzyme titration

Seven samples containing 500 µl of cell suspension (1 million cells/ml) was used. Different treatments were given to the samples (Table 6). Samples 1-3 were controls, while the rest of the samples were treated with the photosensitive ethyl 7-okso-7h-tieno[2,3-A]-quinolisin-8-carboksylate (RO)(Ro 12-9786)(RO)(3µM) (a gift from Dr. Elmar Gocke). This was done in order to induce oxidative DNA-damage by use of a light source.

Table 6. Different treatments with RO, 1100 LUX exposures and lengths of exposure given to the different samples. A positive (+) sign indicates that the sample got the corresponding treatment, and a negative (-) sign indicate that the treatment was not given to the sample. The three first samples, 1-3, were controls.

Sample	RO treatment	1100 LUX exposure	Time (minutes) of LUX exposure
1	-	-	-
2	+	-	-
3	-	+	10
4	+	+	2
5	+	+	4
6	+	+	6
7	+	+	10

When working with RO, double sets of nitrile gloves were used as a safety measure, since the chemical is mutagenic. Since RO is light sensitive the following steps were performed by keeping the samples in the dark. When exposing the cells treated with RO the samples, were held under a light source (1500, cold light)

Embedding the cells on the GelBond film was done as described above. The next day the films were quickly rinsed in distilled water before being put in Collins buffer (50ml/film) at 4°C. After 10 minutes the old Collins buffer was removed and new was added for 50 minutes. While the films were treated in Collins buffer the enzyme buffer was prepared. Collins buffer (at room temperature) was added BSA (0.2 mg/ml) and preheated at 37 °C for approximately an hour.

For the pre-enzyme treatment, the enzyme was thawed and diluted 1:100 in Collins buffer/BSA. Incubation with enzyme lasted for 1 hour with the following final concentrations for FPG; 0, 0.01, 0.1 and 1 µg/µl. Thereafter the same steps are followed as described above (the steps from when the films were placed in unwinding buffer).

When scoring the films and analyzing the data, the optimal enzyme concentration was evaluated to be 1 µg/ml (Figure 14), which was used in the experiment with GA. When using FPG the steps with Collins buffer described under “Enzyme titration” are the same. Also, when using FPG enzyme, a control film was needed without any FPG treatment.

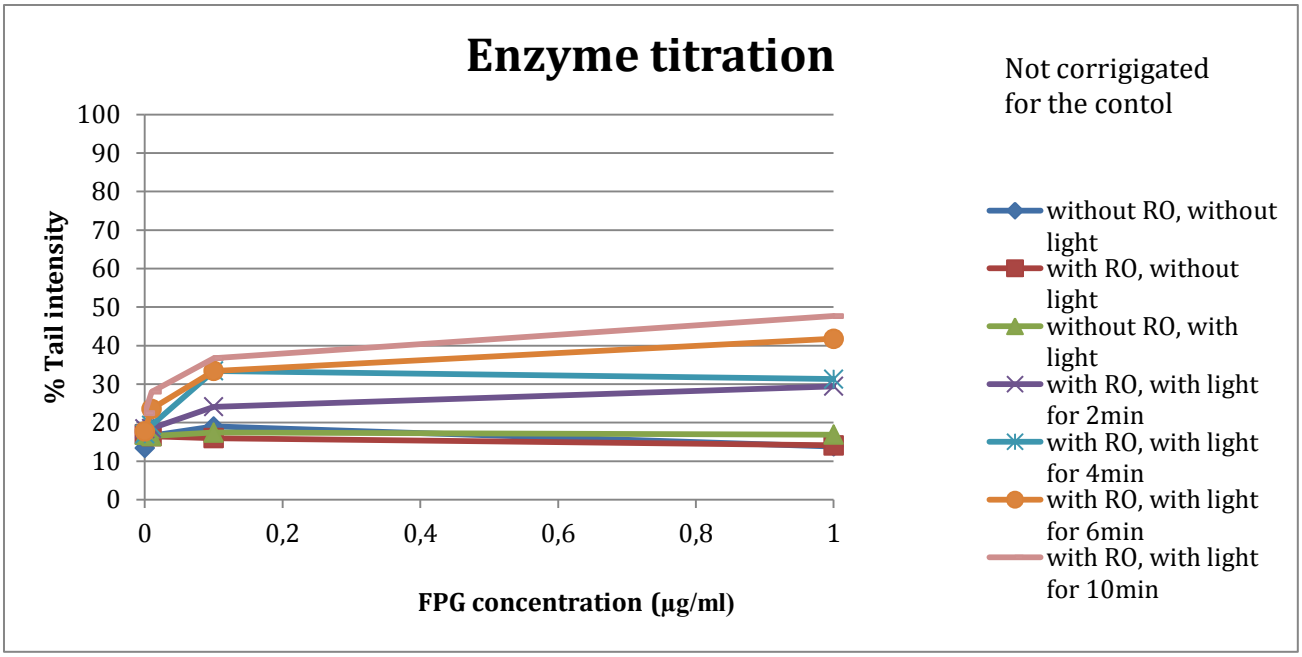


Figure 14. Titration curve of FPG created in a dose dependent manner. Saturation (DNA damage observed) was detected after FPG 0.1µg/ml added. The highest concentration of FPG (1 µg/ml) also detects DNA damage in the saturation part of the curve.

2.5 Statistical analysis

Using statistics, data can be analyzed and one can draw general conclusions based on a limited set of data. One can discriminate between real observations or observations by chance. Data were analyzed using SigmaPlot (SigmaPlot software; Jandel Scientific, Erkrath, Germany). The graphs were also made with this program.

In this study, three identical, independent experiments were carried out, except for the experiment with FA with different time points. Because of time limitations this experiment was only performed two times. Each experiment is considered as a biological replicate. In each sample in the comet assay method, four technical replicates were used. This was mainly done in case of unforeseen challenges with the method.

For each sample 120 single cells were scored, with 30 single cells per replicate. In one of the experiments with FA there was no HEK-293 N750 cells present in the 24h sample. Therefore no statistical analyses have been done on that time.

The median % tail-DNA intensity value was extracted from the raw data for each sample in each experiment. The experiment has more than two groups and two or three factors, and thus, the Two-Way-ANOVA or Three-Way-ANOVA would be an appropriate tests. However, since the sample size is small (n=3), it may be difficult to detect assumption violations such as, non-normality and inequality of variances even when they are present. Some statistical packages include an option for a non-parametric test for the Two-Way-ANOVA. This is not the case for SigmaPlot, and instead a rank transformation has been used on the data before using the standard Two-Way-ANOVA test. Holm-Sidak method was used to identify which groups differed from one another. Significant differences ($p < 0.05$) compared to control is indicated by an asterisk (*).

Conocer and Iman already described the use of ranked data in parametric methods in 1981, and they named this method “The rank transformation approach”. Note that the Kruskal-Wallis test is a rank transformation approach of the One-Way-ANOVA test (Conover WJ August 1981).

Vertical point plot was chosen as the best representation of the data, since it shows the variance in the different experiments. The median of each technical replicate was plotted for each flask. In the text, the results are presented as the calculated means of the medians (of the samples) and the standard error for each group.

3. Results

3.1 Flow cytometry

Cellular measurements by flow cytometric analysis made it possible to assess the individual cells within a population and look at their DNA content. HEK-293K cells and HEK-293 N750 cells were stained with Hoechst 33258 (1 ug/ml), and the amount of apoptotic cells (Sub- G1) in the cell sample, were analyzed.

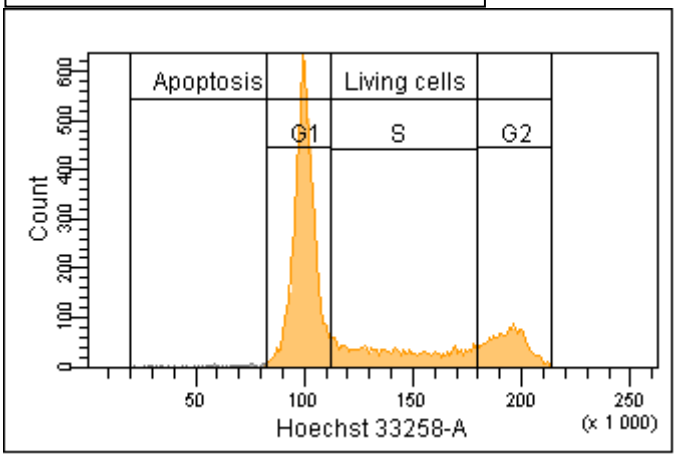
To investigate the cell cycle and state of apoptosis, in the cell lines, flow cytometric analysis was used, and the relative amount of cells in the cell lines were approximately: 63-65 % (G1), 22-25 % (S) and 6-10 % (G2) compared to only 1-4 % (Sub-G1) of the total population (Figure 15).

Then the cell lines got different treatments to examine if the treatment with MMS and /or CPT had an effect on the cell cycle or induced apoptosis, by examining the relative amount of cells in the different parts of the cell cycle (Figure 15).

There were no effects observed on apoptosis for any of the treatments, and also not any difference between the cell lines (Figure 15). Sine there were no effects on apoptosis, only histograms of control treatment as well as the treatment with CTP and MMS (discriminating between the times) is shown. The whole representative presentations from one out of four experiments can be seen in appendix four.

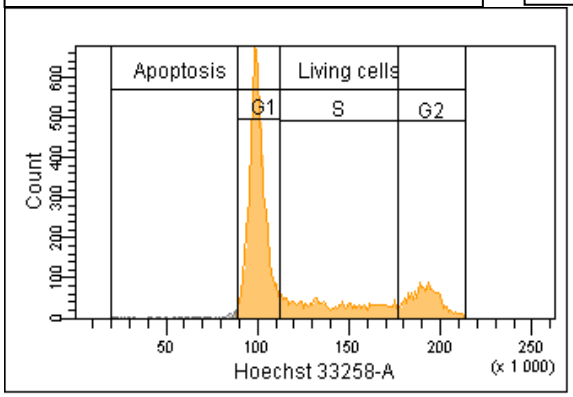
A.1

HEK-293K
Control treatment



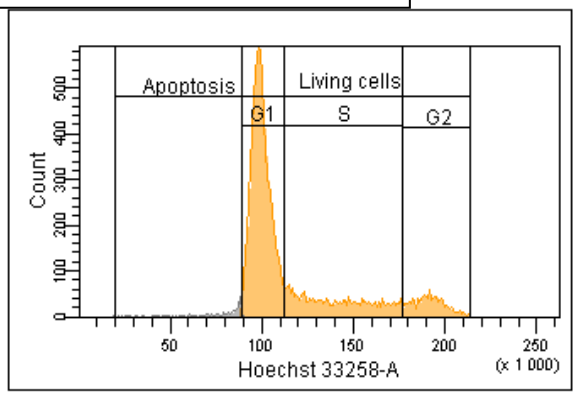
A.2

HEK-293K,
Treatment with CPT (10 µM)
and MMS (600µM)
1 hour



A. 3

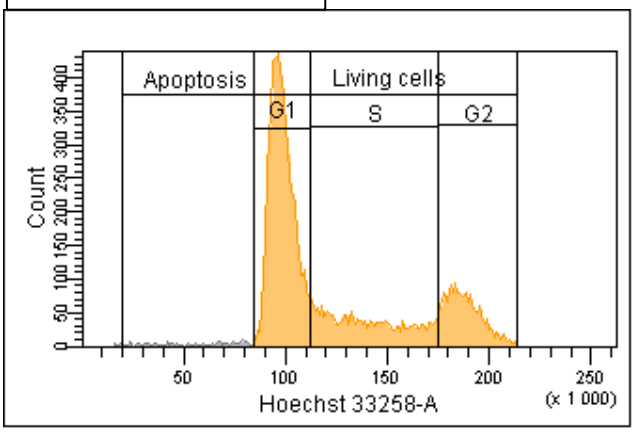
HEK-293K,
Treatment with CPT (10 µM)
and MMS (600µM)
6 hours



Apoptosis:	*Living cells:*		
A.1: 1.67 % ± 0.66	*A.1: 98.00 % ± 0.74*		
A.2: 1.67 % ± 0.66	*A.2: 98.00 % ± 0.74*		
A.3: 2.58 % ± *1.80*	A.3: 97.85 % ± 0.41		
	G1:	S:	G2:
	A.1: 62.50 % ± 1.20	A.1: 22.30 % ± 0.76	A.1: 15.22 % ± 1.90
	A.2: 62.50 % ± 1.20	A.2: 22.30 % ± 0.76	A.2: 15.22 % ± 1.90
	A.3: 67.68 % ± 1.90	A.3: 21.65 % ± 2.40	A.3: 10.68 % ± 0.65

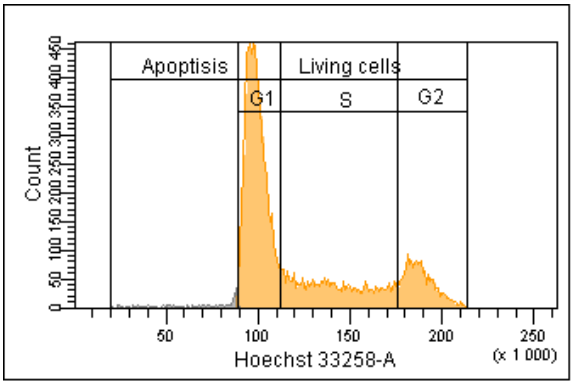
B.1

HEK-293 N750,
Control reatment



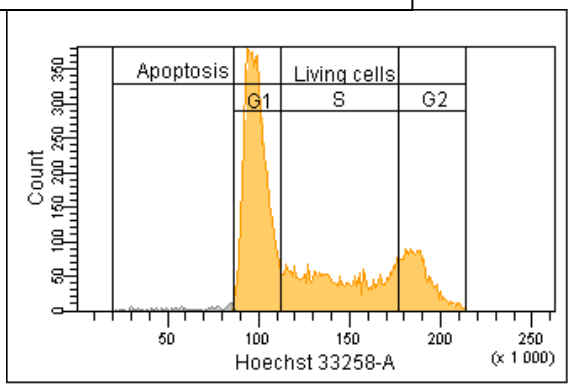
B.2

HEK-293 N750,
Treatment with CPT (10 µM)
and MMS (600µM)
1 hour MMS



B.3

HEK-293 N750,
Treatment with CPT (10 µM)
and MMS (600µM)
6 hours



Apoptosis: *B.1: 3.45 % ±1.35* *B.2:* 2.45 % ± 0.84 *B.3:* 2.8 % ±1.08	*Living cells:* B.1: *96 % ± 1.76* B.2: 97.35 % ± 0.79 B.3: 96.6 % ± 1.24		
	G1: B.1: 61.85 % ± 2.66 B.2: 63.25 % ± 7.36 B.3: 64.58 % ± 4.00	S: B.1: 24.1 % ± 0.84 B.2: 24.75 % ± 4.69 B.3: 26.03 % ± 2.42	G2: B.1: 14.05 % ± 2.67 B.2: 12 % ± 3.09 B.3: 9.43 % ± 3.14

Figure 15. Flow cytometric analysis of HEK-293K (A1-3)) cells and HEK-293 N750 cells (B1-3)). The histograms that are shown represent treatment with CTP (10μM) and MMS (600 μM), from one out of 4 independent experiments. The y-axis shows the number of cells (cell count) and the x-axis shows the DNA content (measured by using Hoechst 33258 (1 μg/ml)). The histograms are divided into apoptosis and living cells. The values in the box under the histograms are quantifications of the flow cytometry experiments. All values shown are the average from the four experiments along with standard deviation.

3.2 Western blotting

Western blotting was performed on the HEK-293 N750 cell line to confirm the expression of the truncated form of the APC gene. The HEK-293K cells, which lack the truncated APC, was used as control. Medium with tetracylcine hydrochloride (0.01μg/ml) was used on the HEK-293 N750 cells to induce truncated APC. Western blotting analysis with antibody (Ab-1), specific to the amino terminus of APC, was used. This antibody will detect both full length and truncated APC. B-aktin was used as a loading control.

The Western blot depicts 3 independent experiments for each cell line. Lane 1-3 represents HEK-293K cells, and lane 4-5 represents HEK-293 N750 cells (Figure 16). A protein with 80 kDa (indicated by an arrow), was the truncated protein expressed by the HEK-293 N750 cells, and was only detected in all of the HEK-293 N750 samples (Figure 16). Furthermore, both cell lines also express an unknown protein in the Western blots, which is likely to be a cross-reaction of an unknown protein with the APC antibody. Since the aim of this experiment was not to quantitate the amounts of APC in HEK-293 N750 cell line, but only to verify the presence of truncated APC, the results related to the loading control β-actin was not discussed.

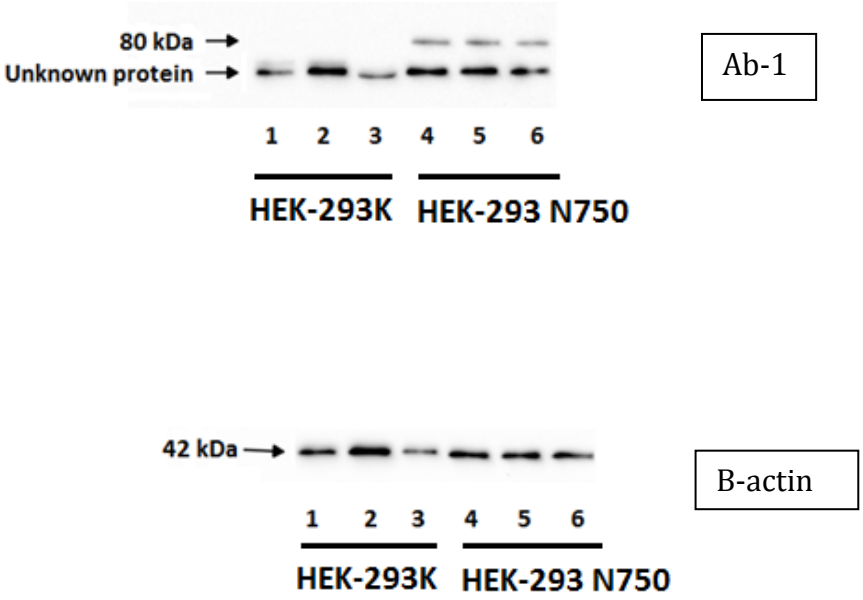


Figure 16. Three experiments with HEK-293K cells (lanes 1-3) and HEK-293 N750 cells (lanes 4-6), was performed to detect expression of the truncated HEK-293 N750 form of the APC gene. The primary antibody being used was Ab-1, and B-actin antibody was used for a loading control.

Western blotting was also used to investigate apoptosis in the HEK-293K and HEK-293 N750 cell. The cells were exposed to CTP (10µM), MMS (600 µM) and a combination (Figure 17 and 18).

Caspases are normally activated by cleavage of the protein when apoptosis is executed, and therefore cleaved- caspase- 3 can be used as a marker for apoptosis. The presence of caspase-3 and cleaved-caspase-3 was investigated using the antibody cleaved- caspase 3. As a loading control B-aktin was used (Figure 17 and 18).

A protein with molecular weight at 35 kDa was detected, which is likely to be the caspase-3 protein. No cleaved-caspase-3 was detected after treatments in any of the cells lines. Western analysis revealed that caspase- 3 is up-regulated in in both cell lines, and in almost all the treatments given (Figure 17 and 18), even though only weak bands could be detected in lane 8, 12 and 13, and even weaker bands in lane 9, 10, 11 and 14.

Figure 17 show higher level of caspase-3 protein in HEK-293K cells in the long time exposure compared to the shorter exposure time, and also higher levels were found in the HEK-293K cells compared to HEK-293 N750. However, due to un-even protein loading, both within lanes with the same cell line and between the cell lines, no firm conclusion can be drawn. Nevertheless, no cleaved-caspase-3 was detected, indicating no induction of apoptosis.

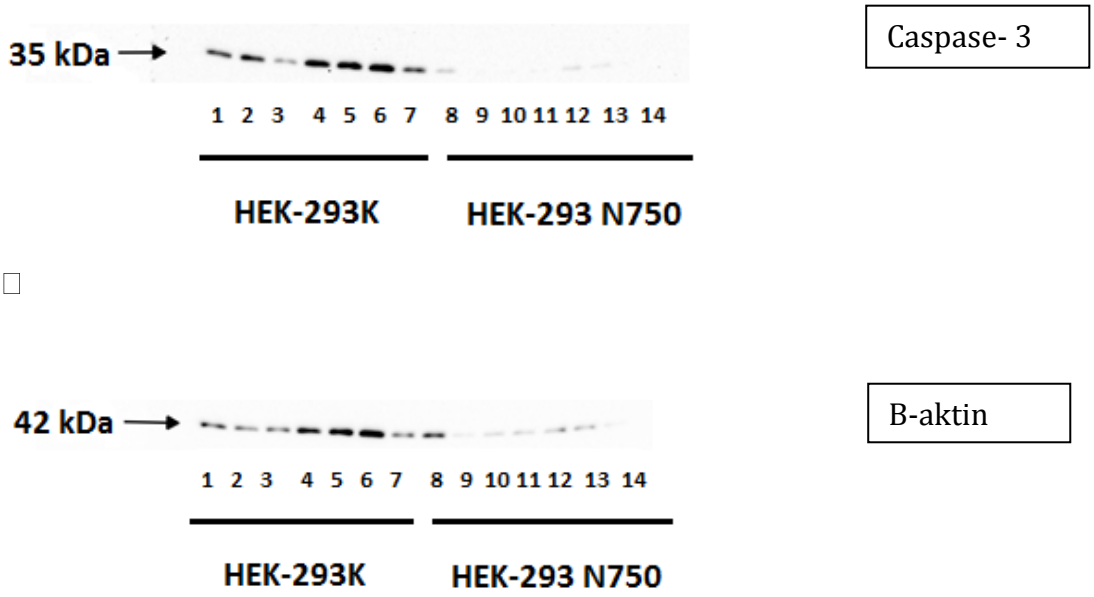


Figure 17. The figure shows representative Western blots of HEK-293K cells (lanes 1-7) and HEK-293 N750 cells (lanes 8-14), using caspase-3 (n=3). Lanes 1-7 are: control (0 mM)/ CPT (10 µM) and MMS (600 µM)/ CPT (10 µM)/ MMS (600 µM)/ CPT (10 µM) and MMS (600 µM)/ CPT (10 µM)/ MMS (600 µM), with the following exposure times: 1 hour/ 1 hour/ 1 hour/ 1 hour 15 minutes/ 6 hours/ 6 hours/ 5 hours 15 minutes. The same order was in lanes 8-14. B-aktin was used as a loading control.

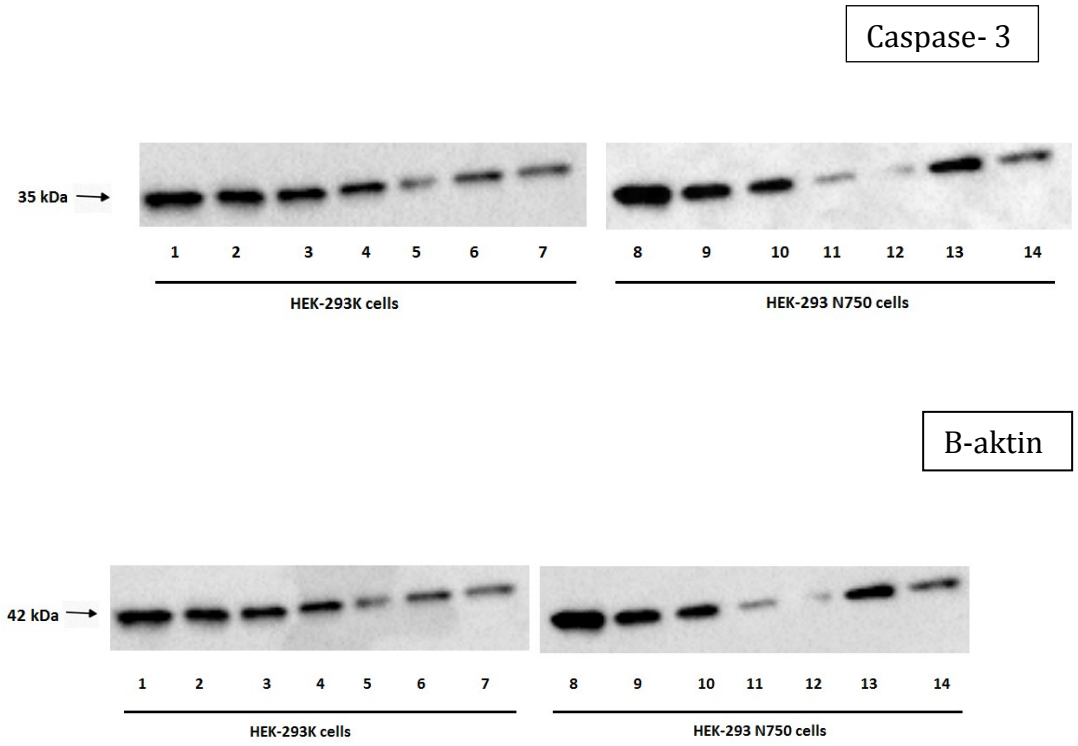


Figure 18. The figure shows representative Western blots of HEK-293 cells (lanes 1-7) and HEK-293 N750 cells (lanes 8-14), using caspase-3 (n=3). Lanes 1-7 are: control (0 mM)/ CPT (10 µM) and MMS (600 µM)/ CPT (10 µM)/ MMS (600 µM)/ CPT (10 µM) and MMS (600 µM)/ CPT (10 µM)/ MMS (600 µM), with the following exposure times: 48 hours 15 minutes / 49 hours / 49 hours / 48 hours 15 minutes/ 49 hours / 49 hours / 48 hours 15 minutes. The same order was in lanes 8-14. B-aktin was used as a loading control.

3.3 Comet assay – testing of genotoxicity

3.3.1 FA induced genotoxicity in HEK-293K cells and HEK-293 N750 cells

The comet assay was used to measure the potential genotoxic effects of FA on the cells, and the experiment was replicated three times. The cells were treated with millimolare concentrations of FA (0, 0.5, 3 and 10 mM) for 3 hours. FA did not induce any detectable genotoxic effects at any tested dose. There was no statistically significant difference between the cell lines (p= 0.881) and also not between the different treatments (p= 0.992) (Figure 19).

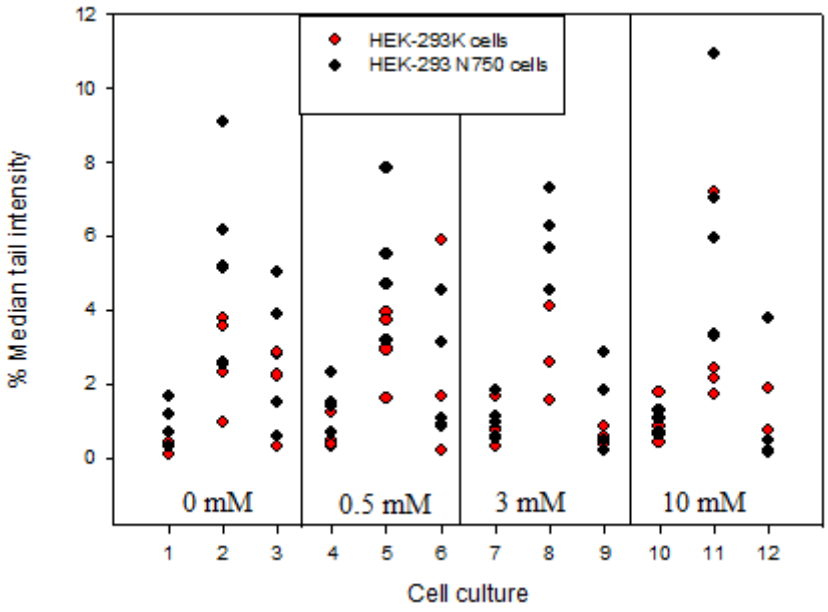


Figure 19. Dose-response curve of FA in the comet assay. The HEK-293K cells and HEK-293 N750 cells were exposed for 3 hours. The x-axis shows the different cell culture (in a flasks) and the y-axis shows % Median Tail Intensity. The vertical lines separate the treatments, with 3 experiments for the same treatment within each box. From left to right the treatments are: 0 mM control (flask 1-3), 0.5 mM (flask 4-6), 3 mM (flask 7-9) and 10 mM (flask 10-12). Each symbol represents the % Median Tail Intensity of one technical replicate.

The highest concentration of FA (10 µM) from the dose-response experiment was used to perform two independent experiment with different exposure times (0, 1, 5, 7 and 24 hours) (Figure 20), to investigate if changes in exposure time would increase DNA damage. Time

did not increase the effect of FA on DNA damage measured by the comet assay (p= 0.473). However, there seems to be a slight difference in DNA damage between the cell lines, where HEK-293K shows a trend to have more DNA damage than the HEK-293 N750 cells, but this is not statistically significant (p= 0.063).

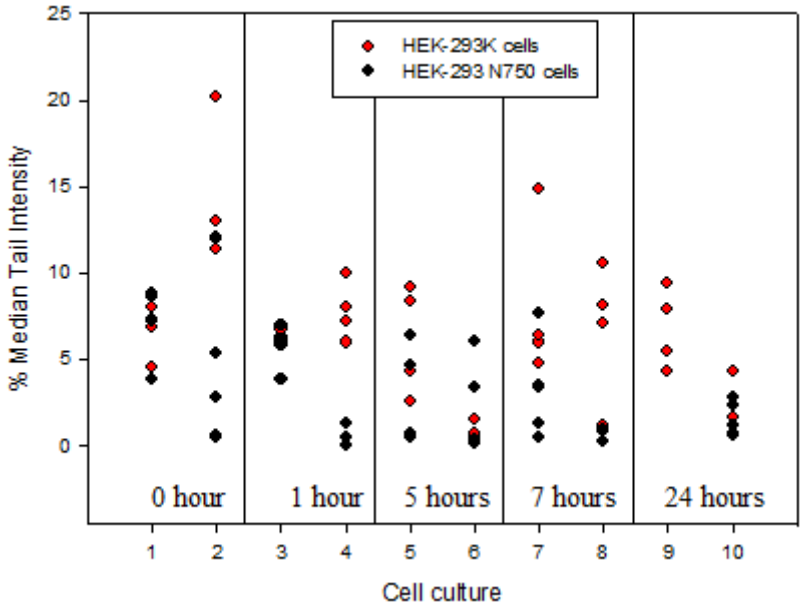


Figure 20. Time-curve with FA in the comet assay. The HEK-293K cells and HEK-293 N750 cells were exposed to 10 mM FA 5 different times. The x-axis shows the different cell culture (in a flasks) and the y-axis shows % Median Tail Intensity. The vertical lines separate the time with 2 experiments for the same treatment within each box. From left to right the treatments are: 0 hour (flask 1-2), 1hour (flask 3-4), 5 hours (flask 5-6), 7 hours (flask 7-8) and 24 hours (flask 9-10). Each symbol represents the % Median Tail Intensity of one technical replicate.

3.3.2 DNA damage induced in HEK-293K cells and HEK-293 N750 cells by GA

The comet assay was used to detect DNA damage after exposure to GA. To assess DNA base modifications we used the comet assay with lesion-specific endonuclease, FPG (1 µg/ml).

In a pilot experiment (not shown) different dose of GA was tested (0.5, 2 and 5mM) for 2 hours. Treatment with FPG at the lowest dose induced 100 % DNA-damage compared to the

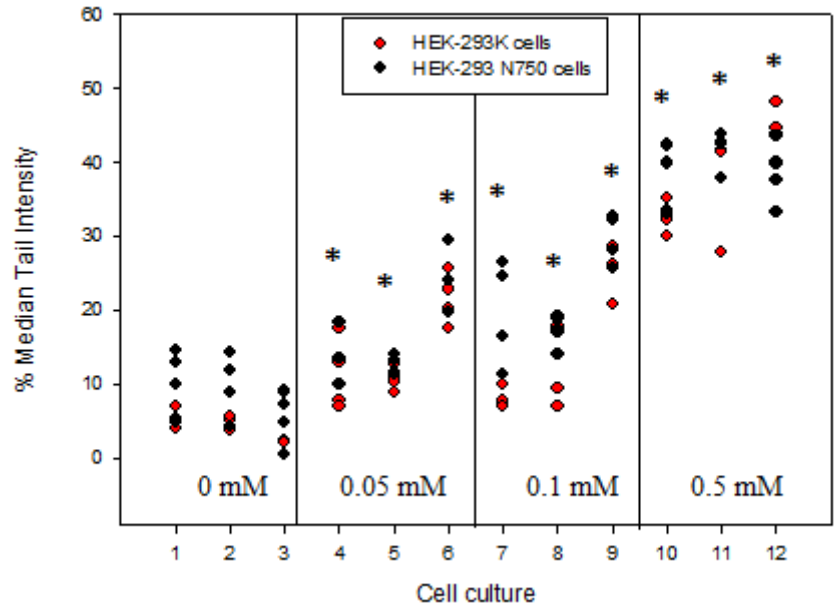
control. This was the case for both cell lines. Since this was a pilot used for further experiments, no statistics was performed.

Based on the pilot, lower doses of GA were used (0.05, 0.1, 0.5 mM) in three independent experiments. After two hours exposure time DNA damage could be detected in a dose dependent manner, with and without the use of FPG (Figure 21).

In the treatment, where FPG was not included, the treatment had a statistically significant impact on the induced DNA damage ($p < 0.001$). For both cell lines increased DNA damage was observed for each dose (HEK-293K lowest to highest dose: 14.14±3.34, 14.91±5.71, 39.14±3.56. HEK-293 N750: 17.00±3.53, 22.02±3.54, 40.19±1.05) compared to the control (HEK-293K: 3.88±0.86 HEK-293 N750: 8.54±1.26). However, there was no statistically significance between the cell lines ($p=0.09$).

Further, when including FPG (1 µg/ml) there was a statistically significant increase in induced DNA- damage ($p < 0.001$). For both cell lines increased DNA damage was observed for each dose (HEK-293K, lowest to highest dose: 66.08±12.80, 68.37±6.35, 97.26±0.58. HEK-293 N750: 59.78±6.23, 69.44±3.84, 96.18±1.08) compared to the control (HEK-293K: 12.73±3.66. HEK-293 N750: 15.24±1.06). However, there was no statistically significance between the cell lines ($p=0.09$).

A. Without FPG



B. With FPG

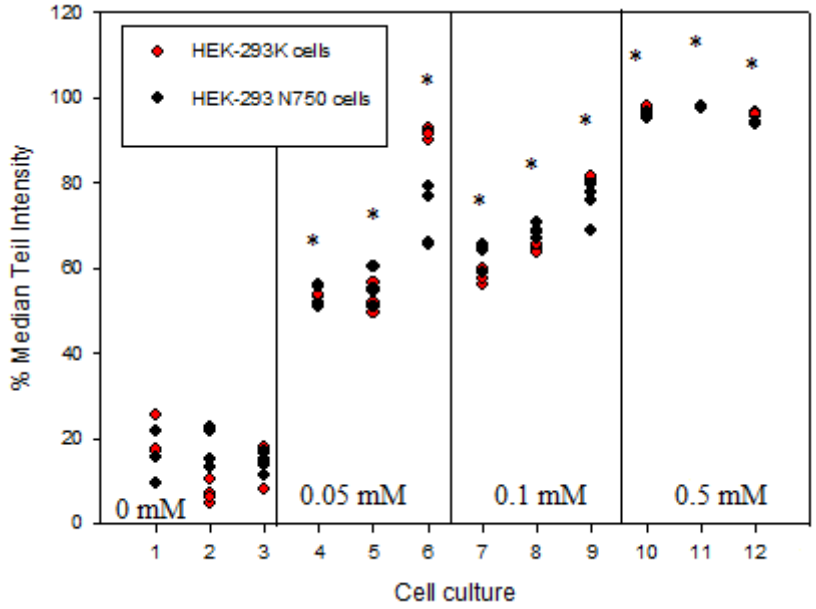


Figure 21. DNA damage induced by millimolar concentrations of GA, measured in the comet assay with FPG enzyme (1 µg/ml) and without. HEK-293K cells and N750 cells were exposed to GA for 2 hours. The x-axis shows the different cell culture (in a flasks), and the y-axis shows % Median Tail Intensity. Each symbol represents the % Median Tail Intensity of one technical replicate. From left to right the treatments are: control (flask 1-3), 0.05 mM (4-6), 0.01 mM (flask 7-9) and 0.5 mM (flask 10-12). **A**.Without FPG. **B**. With FPG.

4. Discussion

In 2004, Tighe and colleagues published an article where they observed that cells transfected with APC mutations could initiate chromosome instability. In that study HEK-293 cells were used, expressing either full length APC or mutants; N750, N1309 and N1087 (Tighe, Johnson et al. 2004). In the described experiments cells transfected with mutant APC, HEK-293 N750 cells, compared to control cells, HEK-293K cells was studied. Western blotting was performed to confirm if the truncated protein was still present in the HEK-293 N750 cells. This was important prior to the experiment so that it would possible to draw conclusions of potential differences between the cell lines based on truncated APC protein compared to normal APC protein.

In the master degree by Heidi Nyland, it was found that MMS induced DNA damage in the comet assay, which was more slowly repaired in the HEK-293 N750 cell line compared to the HEK-293K cells (Nyland 2008). This can be due to reduced DNA repair in cells with DNA damage, or higher apoptosis activity in the HEK-293K cells due to the DNA damage. It was therefore interesting to measure apoptosis in the HEK-293 N750 cell line after exposure to MMS compared to HEK-293K line, as this was not performed in the master degree of Heidi Nyland. In the described experiments DNA damage was also studies in the cell lines.

The cells were exposed to the known mutagenic compound MMS for the apoptosis studies, and to the two food processing contaminants, FA and GA for the comet assay. No effect was observed on apoptosis in cells exposed to MMS, and there were no difference with or without mutated APC. FA did not increase DNA damage in any of the cell lines, while GA increased the DNA damage with equal potency in both HEK-293K cells and HEK-293 N750 cells

4.1 Apoptosis and the influence of truncated APC

APC may play an important role in carcinogenesis, by determine whether cells with DNA damage survive or undergo apoptosis. APC participate in the regulation of Wnt signaling, and the Wnt signaling pathway has been shown to both positively and negatively regulate apoptosis (Benchabane and Ahmed 2009). Therefore, cells with truncated APC may have

impaired apoptosis, and as a consequence be more sensitive to DNA damage, by alkylating agents.

The results from the flow cytometric measurements on apoptosis, measurements in HEK-293K cells and HEK-293 N750, after treatment with CTP alone or in co-exposure with MMS, showed no increase in the level of apoptosis. By using CTP alone and together with MMS it was anticipated a higher level of DNA damage in the HEK-293 N750 cells, since it was reported in an article by Kaina and colleagues (2012), that CTP inhibits TOP1. One of the important functions of this protein is in DNA repair (Tomicic and Kaina 2013).

The results rare not in accordance with the results shown in a master degree by Heidi Nyland (2008), where it was found that MMS induced p53 in the HEK-293 N750 cell line, shown by Western blotting, while the induction in the HEK-293K cells was less evident (Nyland 2008). Since p53 is activated in the early onset of apoptosis, this indicates that some of the cells might go into apoptosis due to DNA damage rather than repair the DNA damages, and that this might be more pronounced in cells with mutations in APC, but this was not observed.

Activation of caspases is responsible for the characteristic morphology of apoptotic cells (Earnshaw, Martins et al. 1999, Roos and Kaina 2006), and cleavage of caspase 3, resulting in cleaved-caspase-3, was used as an indication of apoptosis. Detection of cleaved- caspase-3 was analyzed by Western blotting of proteins from HEK-293 N750 and HEK-293K cells, but only the parent caspase-3 was detected, after exposure to MMS.

This was surprising, since mutagenic anticancer drugs, such as MMS, induce DSB in DNA and thereby leading to apoptosis. The detection of DSB is very rapidly. Low levels of DNA damage trigger DNA repair, while high levels of DNA damage induces apoptosis (Roos and Kaina 2013). The concentration of a drug will influence response of the cells, and often high concentrations induces apoptosis (Sundquist, Moravec et al. 2006). There is a possibility that the used concentrations were too low for detection of apoptosis, but any increase in concentration would not have been realistic. However, the negative results are in accordance with the negative results on apoptosis using flow cytometry, described above.

Thige and colleagues found that cells with expression of an N-APC mutant could induce CIN in a dominant manner. They found that expression of N750 reduced the accumulation of cells

in mitosis following spindle destruction, and they suggested that N750 mutant could compromise the spindle checkpoint. Mitotic spindles are shorter in the cells expressing the N-APC fragments. Expression of N750 alone did not appear to induce CIN (Tighe, Johnson et al. 2004). It is therefore interesting to speculate if the proper terms for expressing apoptosis are present in our experiments.

As an alternative to undergoing apoptosis, necrosis of the cells could have occurred. Apoptosis inducing signals can induce necrosis rather than necrosis, if there is inappropriately high concentrations of a drug concentration (Sundquist, Moravec et al. 2006). Necrosis can also be due to inhibition of mitochondrial respiration or inactivation of caspases (Nicotera, Leist et al. 1999). This type of cell death, will not be detected in the apoptosis measurements, by flow cytometery, and also not by Western blotting if caspases are inactivated.

In cultured cells, induced to undergo apoptosis, indications of apoptotic events occur much sooner than (5-10 hours) than cells within tissues (11-14 hours) (Sundquist, Moravec et al. 2006). Therefore it is possible that MMS induces apoptosis, but the timing for measuring apoptosis was wrong, however, this is just speculations.

4.2 DNA repair and the influence of the truncated APC

Exogenous and endogenous mutagenic agents attack the genomes of all living cells, resulting in the generation of damaged DNA bases. BER is the main pathway for the repair of endogenous abasic DNA damage, and blocking of BER by APC leads to apoptosis (Jaiswal and Narayan 2008). The role of APC in DNA repair was initially discovered by determine its interaction with proliferation cell nuclear antigen (PCNA), which is known to participate in BER. Changes in APC can therefor interfere with DNA repair, and cells, like the HEK-293 N750 cell line, may therefore be more sensitive to alkylating agents, like MMS, or genotoxic compounds from food. DNA alkylating agents, like MMS, have been shown to induce APC, and induced level of APC can block BER and increase the level of DNA damage. The exposure of HEK-293 N750 cells and HEK-293K cells, to MMS, in the comet assay was already studied in the master degree of Heidi Nyland (2008), and showed that MMS induced DNA damage in both cell lines with approximately the same potency (Nyland 2008). DNA alkylating agents induce a variety of DNA lesions, which may have individual contribution to

cytotoxicity and various genotoxic end points, including chromosomal aberrations that are not yet fully understood

However, two other food processing contaminants, FA and GA, were studied in the comet assay, to detect DNA damage after the HEK-293 N750 cells and HEK-293K cells, but only GA increased the DNA damage. The N750 cells were not more sensitive to DNA damage by GA than the HEK-293K cells, indicating that APC do not change the repair of the DNA damages, from GA. This is not in accordance with publications indicating that BER may be involved in the repair of DNA adducts formed by GA (Johansson, Lundell et al. 2005, Pingarilho, Oliveira et al. 2012) since polymorphism in genes important to BER increase the susceptibility to GA induced DNA damage (Pingarilho, Oliveira et al. 2012).

4.3 The genotoxic effects of compounds from heat processed food

Our data on FA, with three different doses, did not show a genotoxic affect on the HEK-293 N750 cells or HEK-293K cells. In another study by Gomez-Arroyo and colleagues (1985), they also did not find any genotoxic effect, when investigating sister chromatid exchange, on human lymphocytes (Gomez-Arroyo and Souza 1985). This is in unity with the results in a study by Mortelmans colleagues (1986) where they tested mutagenicity of FA in four different strains of *Salmonella typhimurium* without any increase in DNA mutations. Another sturdy with *Salmonella typhimurium* showed that small amounts of FA are bioactivated to a mutagenic compound, 2-sulfooxymethylfuran in strains expressing human sulfotransferase 1A1 and in FVB/N mice (Monien, Herrmann et al. 2011). This indicates that FA might need the presence of sulfotransferases to be activated.

FA is much less studied than furan, and most experiments performed with FA are inhalation experiments. Little is known about the potential hazard from oral exposure and concentrations in foods. A 2-year inhalation study performed by the National Toxicology program (NTP) showed that FA induced renal tubule neoplasms in mice and nasal neoplasms in rats. NTP concluded that there was “some evidence of carcinogenic activity” (National toxicology program 1999). However, FA is negative or only weakly positive in several conventional genotoxicy tests (Shinohara, Kim et al. 1986, Aeschbacher, Wolleb et al. 1989).

Also in a study by Stich et al. (1981) they observed that FA had clastogenic activity in Chinese hamster ovary cells (CHO) (Stich, Stich et al. 1981). If FA mainly has clastogenic activity, it cannot be expected to increase DNA damage in genotoxicity test that only detect DNA mutations, like the Ames tests.

In the pilot study with GA there was a significant effect already from the second exposure dose, and the response in the comet assay increased when using FPG. The treatments given to the cells showed a dose-dependent effect relative to the control. Our results are consistent with a study done by Hansen and colleagues (2010) where they found that GA induced DNA damage in mouse and human peripheral blood lymphocytes, at millimolare concentrations. The found a dose-dependent association between the different treatments, and they also found a dramatic effect in the amount of DNA damage when using FPG (Hansen, Olsen et al. 2010). In another study by Besaratinia et al. (2004) they also reported dose-dependent effects of GA, and they found that GA was more mutagenic than acrylamide (Besaratinia and Pfeifer 2004).

However, it is difficult to comparing the results from this study to other studies. This is because nobody has studied the HEK-293K and HEK-293 N750 cells in the comet assay. There are methods for detecting genotoxicity, but comparisons are hard to draw since the different methods detect different kinds of genotoxicity.

5. Conclusion

In this study the effect of MMS on apoptosis in cells with normal APC (HEK-293K) and truncated APC (HEK-293 N750) was investigated.

1. There were no indications that HEK-293K N750 cells were more sensitive towards MMS, and no apoptosis was observed.

The effects of the mutagenic food compounds FA and GA was investigated.

2. FA did not give a genotoxic effect, since no increased DNA-damage was observed. GA gave a dose-dependent increase in DNA-damage.
3. The use of FPG increased the DNA-damage observed for each dose different from the control dose.
4. The HEK-293 N750 cell line was not more sensitive towards mutagenic compounds.

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7. Appendixes

Appendix 1: Products and producers

Product	Producer
Albumin bovine (BSA)	Sigma Aldrich, USA
Aprotinin	Sigma Aldrich, USA
Anti-APC (Ab-1)	Calbiochem, Germany
β- actin	Sigma Aldrich, USA
B- Mercaptoetanol	Sigma Aldrich, USA
CTP	Sigma Aldrich, USA
Cellulose filterpaper	Sigma Aldrich, USA
DC Protein assay	Bio-Rad, USA
DMEM	VWR, USA
DMSO	Merck, Germany
Dry milk	Fluka, Switzerland
EDTA	Sigma Aldrich, USA
FCS	PAR The Cell Culture Company
Glycerol	Sigma Aldrich, USA
Glycin	Sigma Aldrich, USA
HCI	Merck, Germany
Hoechst 33258	Invitrogen, USA
Hyg. B.	Invitrogen, USA
L-Glutamine	Sigma Aldrich, USA
Leupeptin	Amersham Biosiences, Sweden
Metanol	Lab-Scan, Polen
MMS	Sigma Aldrich, USA
Na2EDTA	Sigma Aldrich, USA
NaOH	Merck, Germany
Natrium orthovandat	Sigma Aldrich, USA
Natrium pyrophosfat	Sigma Aldrich, USA
Paraformaldehyd	Sigma Aldrich, USA
PBS	Dulbecco
Pepstatin A	Calbiochem, USA
PMSF(phenylmethylsulfonyl fluoride?)	Sigma Aldrich, USA

P+S	Lonza, Belgium
Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP	Dako, Denmark
Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	Dako, Denmark
SDS	Fluka, Switzerland
Super Signal West Dura Extended Duration Substrate	Thermo Scientific, USA
Sybr®Gold	Invitrogen, USA
Tris-base	Sigma Aldrich, USA
Triton X-100	Sigma Aldrich, USA
Trizma hydrochlorine	Sigma Aldrich, USA
Trypan blue stain	Bio Whittaker, Lonza
Trypsin EDTA	Lonza, Belgium
Tween	Merck, Germany

Appendix 2: Instruments and software

Instruments	Producer
Bio Rad TC10™ Automatic cell counter	Bio-Rad, USA
TC10™ System Counting Slides, bud chambers	Bio-Rad, USA
Bürker counting chamber	Labor Optic
Seri Cycle CO2 incubator	Thermo Electron
25cm2/75cm2 Culture Flasks	Sarstedt
Vacusafe	Integra, USA
Sonicator	Sonic&Materials, USA
Chemi-Doc	Bio-Rad, USA

Software	Producer
BDB FACSDiva™ Software	BD, USA
Gen5	BioTek, USA
ImageLab	Bio-Rad, USA

Appendix 3: Buffers and solutions

Solutions and chemicals that were not sterile from before or autoclaved, were sterile filtered before getting applied to the cells.

Washing buffer

10 mM Tris- HCl buffer	12 g
0.15mM NaCl	80 g
0.1% v.v. Tween 20	10 ml

Fill ut to 100 ml distilled water, adjust to pH 7.6 if necessary.

5x Lysis buffer

0.5 mM Tris- HCl pH 7.5	6 ml
150 nM NaCl	1.32 g
0.5 mM EDTA	300 µl
1 mM EGTA	57 mg
2.4 mM natrium pyrophosphate	167 mg
1 mM natrium orthovandat	27.6 mg
Triton X-100	1500 µl
Distilled water	30 ml

1x Lysis buffer

5x Lysis buffer	0.5ml
10 mg/ml leupeptin	2.5 µl
0.1M PMFS	25 µl
10 mg/ml Pepstatin A	25 µl
10 mg/ml Aprotenin	2.5 µl
Distillated water	1968 µl

Leupeptin 10 mg/ml

Leupeptin (L-2884)	5 mg
Distillated water	500 µl

Aprotenin 10/mg/ml

Aprotenin (A-1153)	10 mg
Distillated water	1 ml

Pepstatin A 10 mg/ml

Pepstatin A (P-5318)	5 mg
DMSO	500 µl

5x SDS/ running buffer for Western

0.5M Tris-HCl, pH 6.8	12,48 ml
SDS	2 g
B- mercaptoetanol	5 ml
1% Bromphenol blue (??)	1 ml
Add distillated water up to	20 ml

0.5M Tris-HCl, pH 6.8

Tris HCl	17.8 g
Tris- Base	1.7g
Add distillated water up to	250 ml, adjust pH to 6.8 is necessary.

10x Transfer buffer

Tris-Base	30.3 g
Glycine	144g
Add distillated water up to	1000 ml

10x Electrophoresis buffer

Tris-Base	30g
Glycine	144g
SDS	10 g
Add distillated water up to	1000 ml

1x Electrophoresis buffer

10x Electrophoresisbuffer	100 ml
Distillated water	900 ml

1x Transfer buffer

10x Transferbuffer	100 ml
Methanol	200 ml
Distillated water	700 ml

1% dry milk

Skim milk powder	5g
Add washing buffer to	500 ml

3% dry milk

Skim milk powder	15g
Add washing buffer to	500 ml

PBS with EDTA

PBS without Ca^{2+}/Mg^{2+}	500ml
10mM Na_2EDTA	1.86g

Adjust to pH to 7.4 is necessary.

Lysis stock

Distilled water	3L
2.5M NaCl	730.5g
100mM Na_2EDTA	186.0g
10mM Trizma base	6.0g
34mM NaOH	37.5g
34mM N-Lauroylsarcosine sodium salt	50g

Ad distilled water up to 4.45L, and adjust pH to 10, with either HCl or NaOH.

Electrophoresis stock

Distilled water	4L
3M NaOH	600g
0.01M Na_2EDTA	18.6g

Add distilled water to 5L

Lysing solution

Stock	300 ml
DMSO	33, 3 ml
Triton X-100	3, 33 ml

Electrophoresis solution (10x)

Stock	200 ml
HCl (37%)	12 ml
Distilled water	1.8L

Collins buffer

Distilled water	4L
40mM Hepes	47.65g
0.1M KCl	37.25g
0.5mM Na_2EDTA	0.93g

Add distillated water up to 5L, and adjust the pH to 7.6, with 7M KOH

Collins buffer with BSA (0.2 mg/ml)

Collins buffer	200ml
40 mg/ml BSA	1 ml

Neutralization buffer (0.4M)

Distilled water	4L
0.32M Tris-HCl	254g
0.08M Tris-base	47.2g

Add distilled water to 5L, and adjust the pH to 7.5, with either HCl or NaOH.

Na_2EDTA stock

0.2M Na_2EDTA	7.5g
Distilled water	100ml

Tris-HCl stock

0.5M Tris-HCL	39.4g
Distilled water	500ml

TE buffer

Distilled water	0.8L
0.2M Na_2EDTA (stock)	5ml
0.5M Tris-HCl (stock)	20ml

Adjust the pH to 8.0

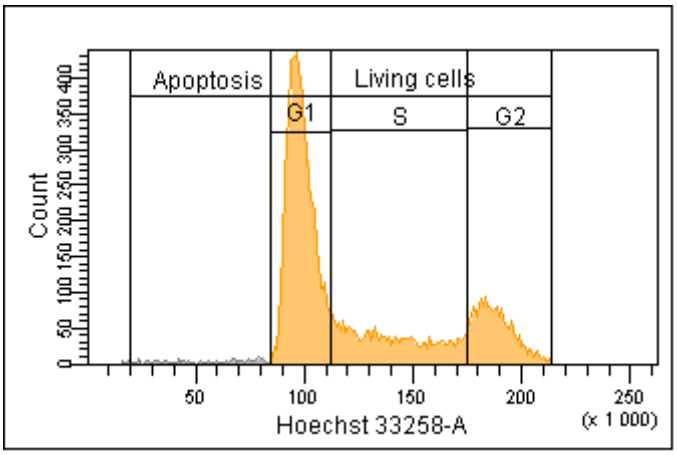
Add distilled water to 1L, adjust to pH 5.8 is necessary.

Dulbecco's PBS

KCl	0.2 g
KH_2PO_4	0.2 g
NaCl	8 g
Na_2HPO_4	1.15 g

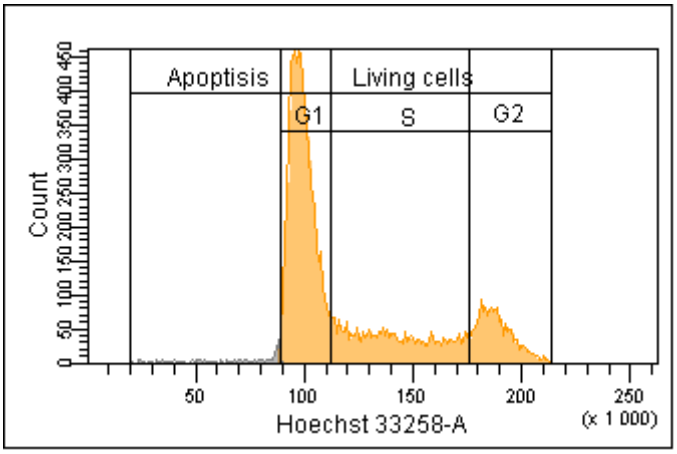
Adjust pH to 7.4

Appendix 4: Flow histograms



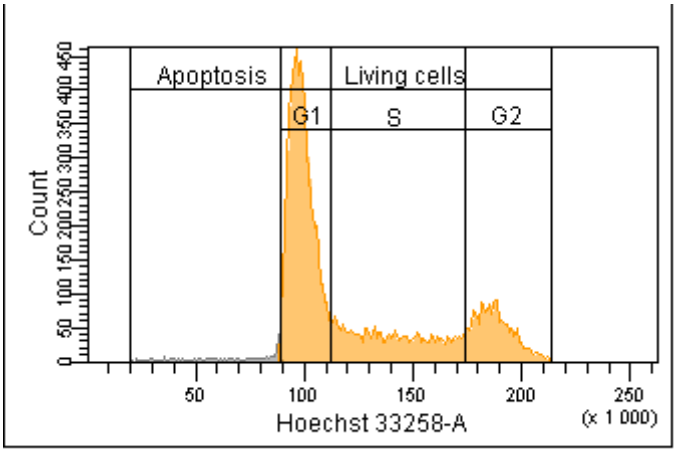
HEK-293 N750.
Control

Apoptosis: 3.45 % ±1.35	*Living cells: 96 % ± 1.76*		
	G1: 61.85 % ± 2.66	S: 24.1 % ± 0.84	G2: 14.05 % ± 2.67



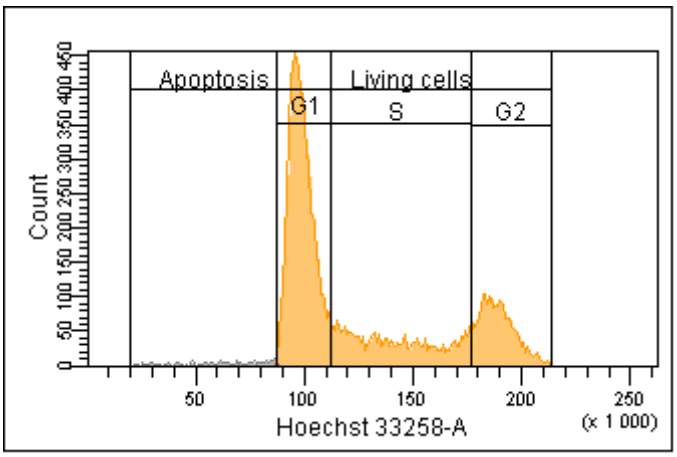
HEK-293 N750.
Treatment with Camp. (10 µM) and MMS (600µM)
1h MMS

Apoptosis: 2.45 % ± 0.84	Living cells: 97.35 % ± 0.79		
	G1: 63.25 % ± 7.36	S: 24.75 % ± 4.69	G2: 12 % ± 3.09



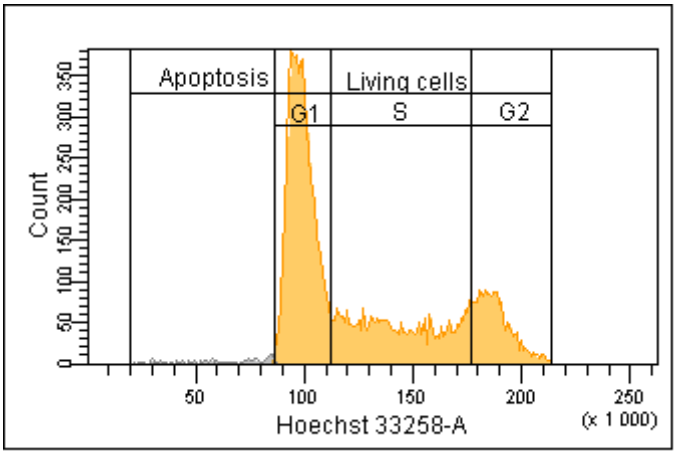
HEK-293 N750.
Treatment with Camp. (10 µM) 1h

Apoptosis: 2.75 % ± 0.85	Living cells: 96.85 % ± 1.03		
	G1: 60.18 % ± 6.80	S: 28.13 % ± 4.61	G2: 11.7 % ± 4.20



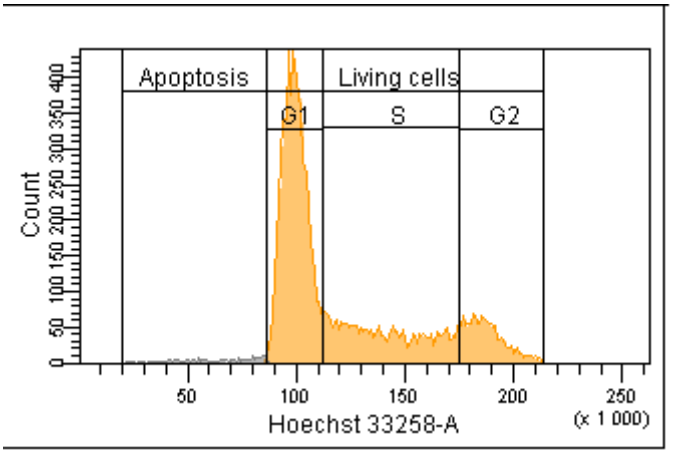
HEK-293 N750.
Treatment with MMS (600µM) 1h and 15 minutes

Apoptosis: 2.28 % ± 0.92	Living cells: 97.4 % ± 0.89		
	G1: 56.08% ± 1.14	S: 29.7 % ± 4.62	G2: 14.25 % ± 3.85



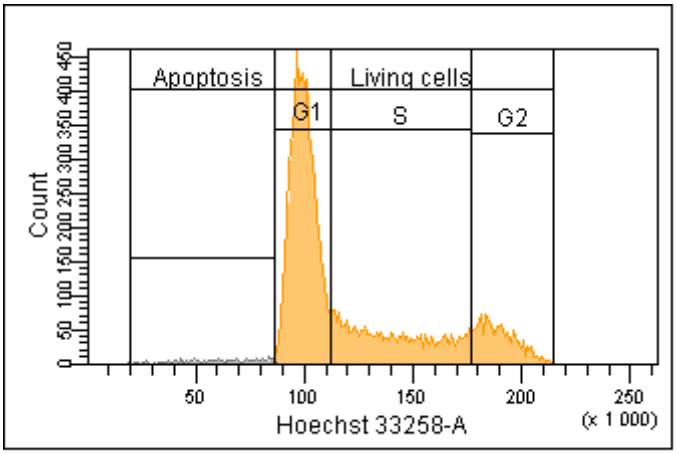
HEK-293 N750.
Treatment with Camp. (10 µM) and MMS (600µM)
6 hours

Apoptosis: 2.8 % ±1.08	Living cells: 96.6 % ± 1.24		
	G1: 64.58 % ± 4	S: 26.03 % ± 2.42	G2: 9.43 % ± 3.14



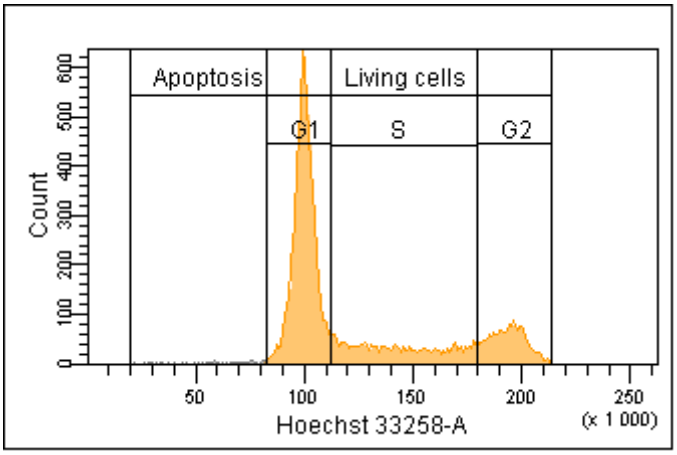
HEK-293 N750.
Treatment with Camp.
6h

Apoptosis: 3.2 % ± 1.86	Living cells: 96.34 % ± 2.22		
	G1: 62.8 % ± 3.34	S: 26.85 % ± 1.38	G2: 10.35 % ± 2.16



HEK-293 N750.
Treatment with MMS (600µM)
5h and 15 min

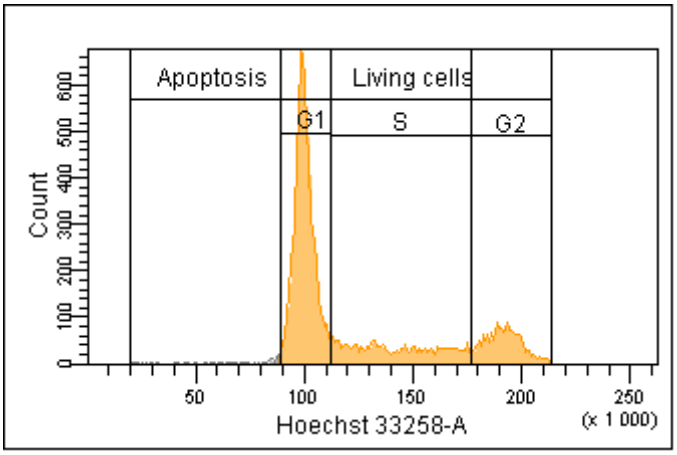
Apoptosis: 3.08 % ±2.07	Living cells: 96.45 % ± 2.16		
	G1: 58.48 % ± 8.45	S: 28.65 % ± 6.21	G2: 13.12 % ± 3.12



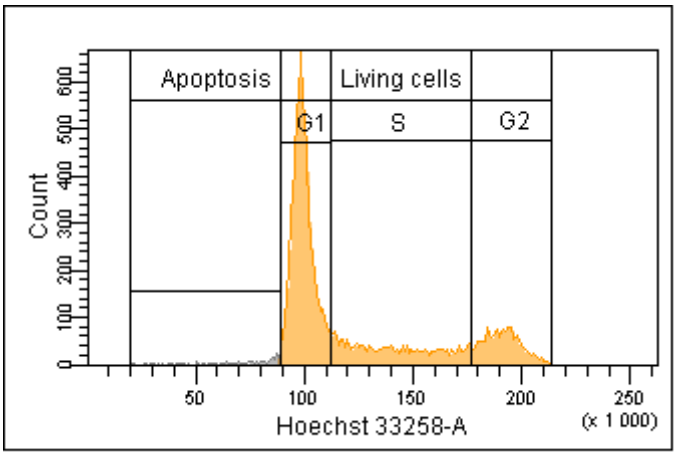
HEK-293K.
Control treatment

Apoptosis: 1.67 % ±0.66	*Living cells: 98 % ± 0.74*		
	G1: 62.5 % ± 1.2	S: 22.3 % ± 0.76	G2: 15.22 % ± 1.90

HEK-293K.
Treatment with Camp. (10 µM)
and MMS (600µM)
1h

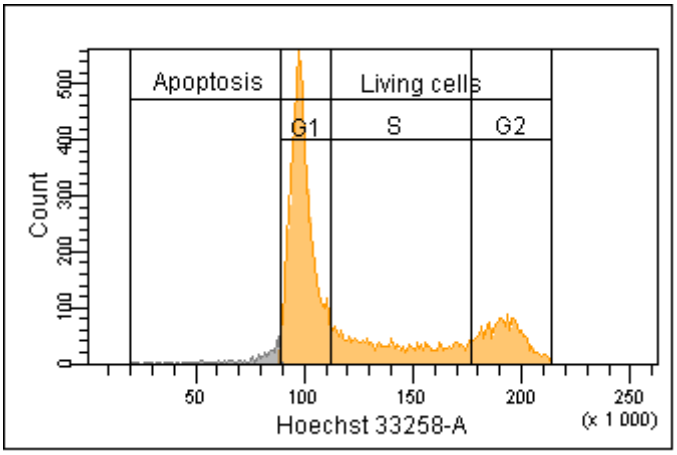


Apoptosis: 1.67 % ±0.66	*Living cells: 98 % ± 0.74*		
	G1: 62.5 % ± 1.2	S: 22.3 % ± 0.76	G2: 15.22 % ± 1.90



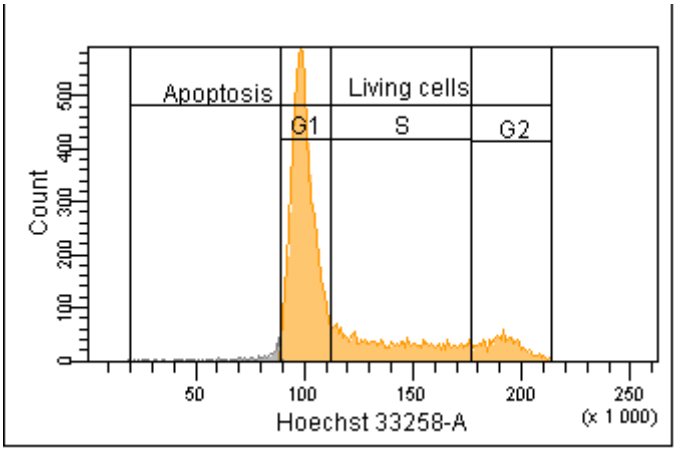
HEK-293K.
Treatment with Camp. (10 µM)
1h

Apoptosis: 2.13 % ± 0.91	Living cells: 97.88 % ± 0.36		
	G1: 65.35 % ± 4.43	S: 21.2 % ± 2.02	G2: 13.45 % ± 2.87



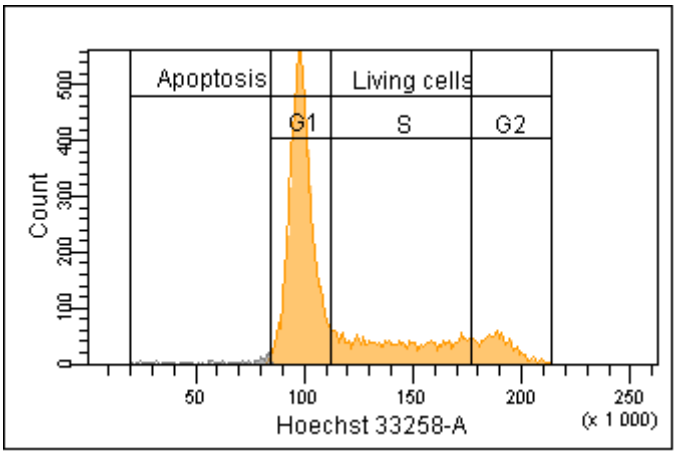
HEK-293K.
Treatment with MMS (600µM)
1h and 15 min

Apoptosis: 2.13 % ± 0.91	Living cells: 97.88 % ± 0.36		
	G1: 65.35 % ± 4.43	S: 21.2 % ± 2.02	G2: 13.45 % ± 2.87



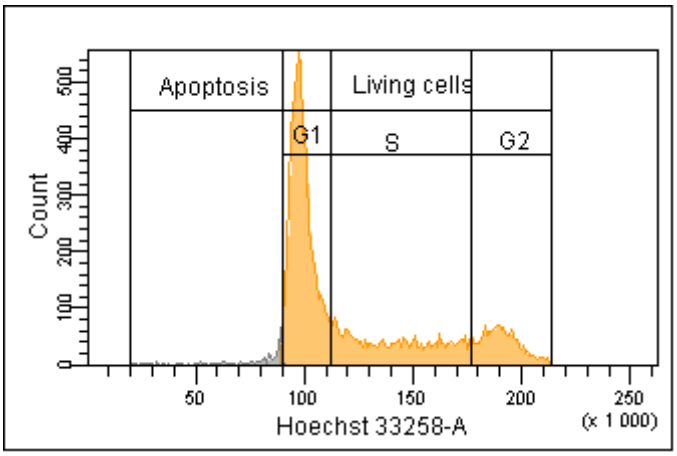
HEK-293K.
Treatment with Camp. (10 µM)
and MMS (600µM)
6h

Apoptosis: 2.58 % ±1.80	Living cells: 97.85 % ± 0.41		
	G1: 67.68 % ± 1.90	S: 21.65 % ± 2.40	G2: 10.68 % ± 0.65



HEK-293K.
Treatment with Camp. (10 µM)
6h

Apoptosis: 2.8 % ± 2.30	Living cells: 97.65 % ± 0.66		
	G1: 66.03 % ± 1.07	S: 23.98 % ± 0.57	G2: 10 % ± 1.11



HEK-293K.
Treatment with MMS (600µM)
5h and 15 min

Apoptosis: 2.8 % ± 2.30	Living cells: 97.65 % ± 0.66		
	G1: 66.03 % ± 1.07	S: 23.98 % ± 0.57	G2: 10 % ± 1.11